STRUCTURAL AND SYNTHETIC STUDIES IN MODIFIED OLIGONUCLEOTIDES

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28 February 2002

CERTIFICATE

This is to certify that the work presented in this thesis entitled "Structural and synthetic studies in modified oligonucleotides" has been carried out by Miss. Vibha Shukla. She has fulfilled the requirement of University of Allahabad, Allahabad, regarding the prescribed period of investigational work for the award of D. Phil. degree.

The work included in this thesis is original and genuine.

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Dedicated
To my parents....

....Who have been my inspiration and who have inculcated in me the spirit to never stop learning and to achieve the highest level in everything I do.

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V. Shukla

Vibha Shukla

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Abbreviations

A^0	: Angstrom Units
A	: Adenosine
Ac	: Acetyl
AIDS	: Acquired Immuno Deficiency Syndrome
Anhyd.	: Anhydrous
Aq. / aq.	: Aqueous
C	: Cytosine
^0C	: degree centigrade
CDI	: Carbonyl diimidazole
Conc.	: Concentrated
cm	: Centimetre
CPG	: (LCAA-CPG) Long Chain Alkylamine - Controlled Pore Glass (resin)
d	: Deoxy
DCC	: Dicyclohexylcarbodiimide
dil.	: dilute
DMT / DMTr	: Dimethoxy trityl
DNA	: Deoxyribonucleic acid
G	: Guanosine
h	: Hour
HIV	: Human Immunodeficiency Virus
HPLC	: High Performance (Pressure) Liquid Chromatography
M	: Mole
mM	: millimole
MMT	: Monomethoxytrityl
min	: minutes
ml	: millilitre
mol / L	: Mole / Litre
nm	: Nanometer
OD	: Optical Density
Odn / Oligos	: Oligonucleotides
P	: Polymer Support
Py.	: Pyridine
R_f	: Retention Factor
RF	: Relative Fluorescence
r. t.	: room temperature
TCA	: Trichloroacetic acid
T	: Thymine
THF	: Tetrahydrofuran
TLC	: Thin Layer Chromatography
TPST	: Tri-isopropylbenzenesulphonyl tetrazole
Tr	: Trityl
UV	: Ultra Violet Spectroscopy
ε	: (epsilon) Molar Extinction Coefficient
λ	: (lambda) Wavelength
μ	: (mue) Micron
μL	: Microlitre
μM	: Micromole
π	: (pi) Electronic Energy Level
σ	: (sigma) Electronic Energy Level
PNA	: Peptide nucleic acid

CHAPTER-1

Introduction & Background

1. Introduction:

Since the elucidation of double helical structure of DNA (deoxyribonucleic acids) the basic genetic material of life. The properties of this remarkable molecule have fascinated scientist all over the world. Tremendous efforts have been devoted to understand DNA structure and function in biology as well as from a chemical and physiological standpoint [1]. Yet very simple just built from four nucleobases[2] adenine (A), guanine (G), cytosine (C) and thymine (T) assembled via a polymer backbone composed of deoxyribose phosphodiesters, this molecule holds the key of life on earth and biological evolution. Part of the secret is the nucleobase molecular complementarity, antiparallel DNA strand to form a double helix. This also ensures high fidelity information transfer as written by the linear sequence of the nucleobases during synthesis of a new DNA copy (replicon), and during transcription of the DNA into cell's messanger molecule. RNA is finally translated to 'work molecule of cell', the protein. Some very fundamental questions still remain to be answered are,

- Why has nature 'chosen' / settled on DNA as the central genetic material of life?
- Is DNA the only possibility or could other chemical structures fulfill the requirements and support (other forms of) life?
- What makes DNA such a good candidate in terms of structure, stability and molecular recognition?

We will most probably never get the final answer to all these questions, but studying the DNA will of course bring us closer to this. Also studies of DNA analogues and mimics may help to shed light on these questions. Furthermore, oligomers of both synthetic DNA and DNA analogues are finding widespread application within molecular biology, genetic diagnostics and gene therapeutic medicine[3-8].

1.1 Synthetic oligonucleotide:

Why synthetic oligonucleotides?

- Researchers have been attempting to synthesise DNA for many years, originally in an effort to understand the relationship between the structure and function.
- As original DNA samples were isolated from biological samples there was no control over the sequence of the DNA.
- Synthetic DNA allows control over sequence order.

- Large amount of DNA can be produced for therapeutic and diagnostic purpose such as antisense and antigene therapy.

Synthetic oligonucleotides containing rational modification have emerged as new tools for practical application in therapeutics and diagnostics apart from their utility in studying molecular recognition of DNA and its interaction with ligand. Chemical modification at nucleobases by tethering of 'intelligent' chemical functionalities has led to construction of designer oligonucleotide of tailored application. The duplex and triplex derived from these have potential advantages over natural DNA in terms of complementation, stability and membrane permeability. Short synthetic oligonucleotides as highly specific inhibitors of transcription and translation have shown great promise as antisense and antigene therapeutic drugs. The quest for oligonucleotide analogues and mimics with improved properties in terms of nuclease resistance and target affinity have led to the development of a large number of DNA analogues.

1.2 Oligonucleotides synthesis:

Michelson and Todd achieved the first successful synthesis of a small piece of DNA in 1955[9]. The major developmental thrust in oligonucleotide synthesis however came from the work of Khorana *et al*[10-15] in 1960's. Following this, chemical synthesis of a repetitive DNA duplex[16-18], of a gene[19,20] and a regulated gene[21,22] was carried out. Khorana *et al.*[23] in 1965 carried out total synthesis of DNA duplex corresponding to tyrosine t-RNA precursor. Since then a number of methods for oligonucleotide synthesis have been developed and perfected. The advent of solid-phase synthesis and automation techniques has made synthesis of an oligonucleotide of a defined sequence easier and facile[24,25]. The following sections give a brief account of various approaches used for oligonucleotide synthesis

The DNA synthesis was originally carried out in solution phase using phosphodiester chemistry which took very long time and was low yielding. The other strategies were used applying the synthesis on solid support has its benefits and also limitations.

Advantages of solid phase synthesis

- The reason is easy to wash.
- Control over sequence composition possible due to stepwise addition.
- Can be automated.
- Efficiency of each addition can be evaluated.

laboratories for large-scale synthesis of oligonucleotides[34,35]. The key point in this approach is that the highly reactive internucleotide phosphate anions are masked as triesters. A schematic representation is given in Fig. 1.2

The protection of internucleotide bond as triesters masks the phosphate group and thus prevents unwanted side reactions. This coupled with the use of solvent extraction and silica gel chromatography increases the yield manifolds. This process is however also associated with certain disadvantages like, side products associated with the condensing reagents[36-38]. This involves sulphonylation of primary hydroxy groups and base substitution/modification reactions, which are poorly understood. Another problem is routine isolation of homogeneous intermediates free of starting materials and side products. Moreover, it is not always possible to use silica gel columns especially when a reaction does not go to completion.

To overcome these problems Narang *et al*[39] modified the "one-pot" triester approach of phosphorylation and coupling[28] to a "two-step" sequential coupling process. Here, the synthesis is started from fully protected mononucleosides, containing a fully masked 3-phosphate group (Fig. 1.3). Since the resulting intermediate contains a fully masked 3'-phosphate group, the necessity for phosphorylation at each condensation step is eliminated. Similar improvements were suggested by Crams *et al*[40]

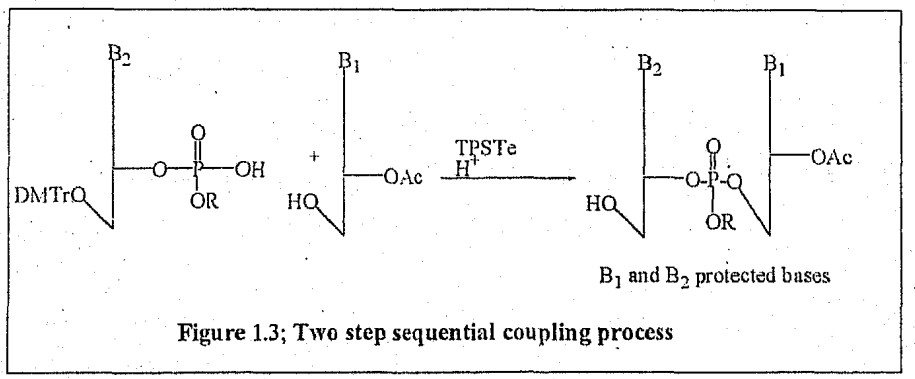

Figure 1.3; Two step sequential coupling process

1.2.(c). The Phosphite-Triester Approach

This method was also introduced by Letsinger *et al*[41]. This involves the coupling of nucleosides by reaction with a phosphodichlorite (a trivalent phosphorous). The method has a number of advantages viz the protecting groups used in oligonucleotide synthesis are stable to phosphodichlorites[41], short time is required for the completion of reaction, low temperature is used

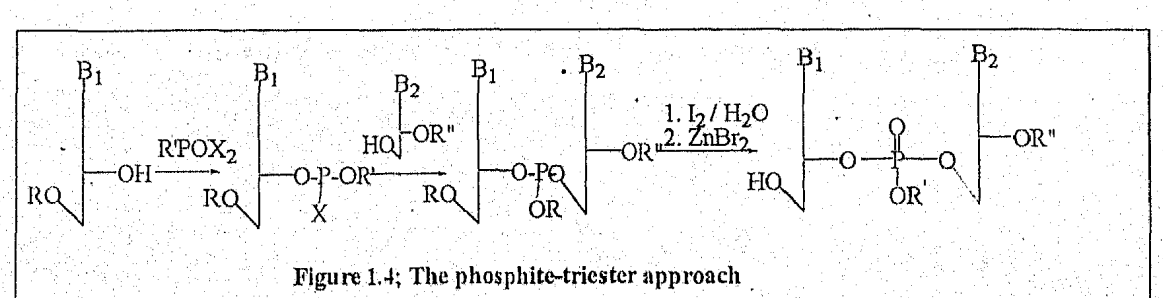

Figure 1.4; The phosphite-triester approach

for condensation phosphochlorite reagents show high selectivity and favourable yields (Fig. 1.4).

Since the reactions involved have fast kinetics, the method is quite suitable for adaptation to polymer supported oligodeoxynucleotide synthesis. In this method the formation of internucleotide bond involves a reaction between deoxynucleoside/oligodeoxynucleotide attached to a polymer support (via free 3'-OH) and a suitably protected deoxynucleoside phosphate. The final steps are capping of unreacted deoxynucleoside/deoxyoligonucleotide and oxidation of phosphite to phosphate bond using I_2/H_2O or lutidine/THF.

Capping is a necessary step used to prevent the formation of several deoxyoligonucleotide chains with heterogenous sequences and is accomplished by using a large excess of very reactive phosphite e.g. diethoxy triazoyl phosphine which would react with deoxynucleoside / oligodeoxy - nucleotide to produce a 5'-diethylphosphite, a relatively non-hydrophobic triester. Since purification involves reverse phase HPLC, all non-hydrophobic failure sequences can be separated from the sequence containing hydrophobic 5'-O-dimethoxytrityl group. The oxidation is sufficiently mild[41] and hence does not generate side products. Seliger[43], however, suggested that oxidation after each elongation cycle increases the overall yields.

Since the high reactivity of phosphite intermediate impairs the selectivity of internucleotide bond formation, it is almost essential to keep the growing chains locked to a polymer support[44]. The automation of this process was reported in 1981[44-46].

1.2.(d). The Phosphoramidite Approach

In this process also, an intermediate compound with phosphorous in trivalent stage is used, which in due course of time, is oxidised to a pentavalent phosphorous leading to a phosphate bond. The reactions involved are therefore very fast and give fairly good yields even in milligram quantities. This approach was introduced by Beaucage and Caruthers[48-49], is very suitable and frequently used now a days[47].

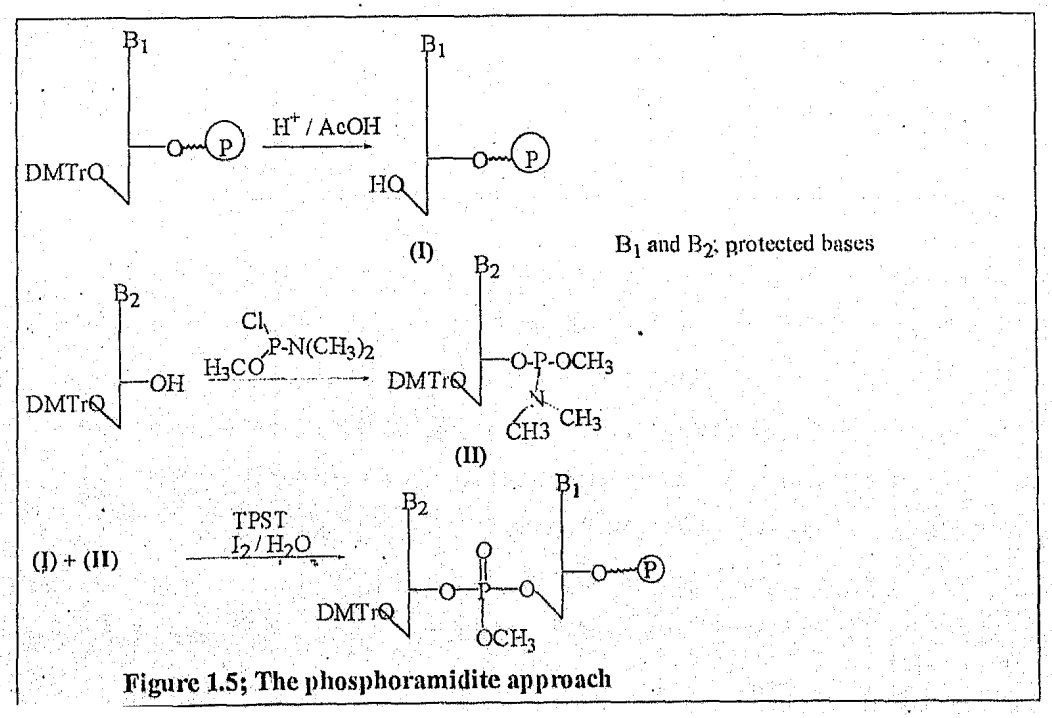

Figure 1.5; The phosphoramidite approach

The process involves adding mononucleotides sequentially to a nucleoside covalently attached to an insoluble solid support. Extra reagents, starting materials and side products are removed by filtration. The condensation step is followed by a series of further reactions like blocking, oxidation, and detritylation, which are performed heterogeneously. Washing and filtration procedures are necessary after each reaction step in order to remove the mobile reactants. This series of consecutive reactions including washing/filtration steps is called "reaction cycle" which has to be carried out for addition of each nucleotide unit to the support. A general strategy for synthesis has been shown in Fig. 1.5.

In this approach deoxynucleoside phosphoramidites are used as condensing agents. Other phosphoramidite derivatives of morpholino or diisopropyl have also been used[48,49]. These reagents are stable enough and can be stored for long time as white solids. These when mixed with various weak acids in acetonitrile, are converted to tetrazolides which reacts rapidly (less than 5 minutes) with yields 90-100% in case of dinucleotides and hence of great use[50-54].

1.2.(e). The Hydrogen-Phosphonate Approach

The higher reactivity of P (III) intermediates in phosphite-triester and phosphoramidite approaches make these methods more desirable in oligonucleotide synthesis. However there has to be a compromise between stability and reactivity and precisely this effort has been made in this approach i.e. to combine the reactivity of P (III) intermediates with the stability of H-phosphonates[55-59]/methylphosphonates[60] (a modification of hydrogen-phosphonate approach).

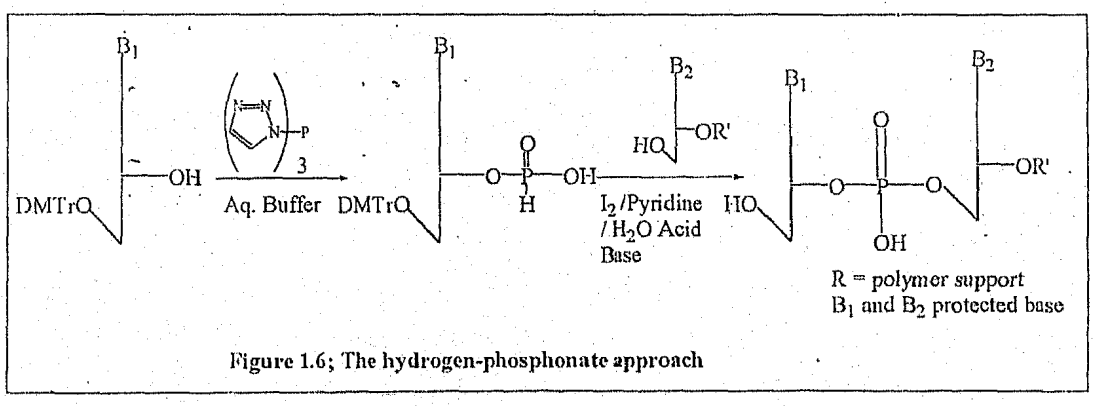

Figure 1.6; The hydrogen-phosphonate approach

In this method, phosphonates are prepared by reacting the appropriately protected nucleoside with tris (1, 2, 4-triazolyl) phosphite followed by hydrolysis. Trimethylacetyl chloride (pivaloyl chloride) is the condensing reagent used for internucleotide bond formation. The oxidation with 2% iodine in pyridine/water[61] is carried out after each step to convert P (III) to P (V) (Fig. 1.6).

The key feature of this approach appears to be the enhanced stability of monomer and the final product. The need for phosphate protection is eliminated and these do not involve activation by coupling reagents. Less rigorous requirement for anhydrous condition and capping makes this approach another popular alternative for oligonucleotide synthesis[62].

1.3 Modification in oligonucleotides to improve stability:

1.3.(a) Base modification

Base modifications can be carried out in two fashions to increase duplex stability[63]. They can be either attempt to increase strength of H-binding found within the base pairs or they can alter the base stacking and groove interactions of the DNA[64].

(i) Alteration of H-Bonding

As one base pair, GC, has the maximum number of H-bonds permitted for structures there is only one base pair, AT, that can be enhanced. The thymine has three sites fore H-bonding. Whereas the A has only two sites. Therefore, the H-bonding ability of bases pairing to thymine must be altered. This is done by substituting diaminopurine[65,66], DAP, for adenine to form a base pair with three H-bonds (synthetically the starting material is G not A). the Tm for oligo containing DAP rises approx. 1^0c / DAP residue. The absolute amount of increase in stability depend the effect of neighboring base pairs.

As previously stated the base stacking provides a large degree of stability[67] for a duplex once the hybridisation has occurred. Base stacking involves π-π interaction with the base above and below the base in question within the same strand. By improving the amount of overlaping of the bases we can improve duplex stability. This can be achieved by introducing extra p- system capable of stacking yet not interfering with the H-bonding specificity of base.

The pyrimidines can be easily modified at the 5- position thus still allowing normal Watson-Crick base pairing. The purine can also be modified at 7 or 8 position ,Unfortunately large groups at the 8-position of the purine disrupt the duplex therefore rendering modification at this position useless. Instead the Nitrogen at the 7-position is replaced by a C to form 7-deazapurine[68]. Which provides a position suitable for modification yet does't disrupt the duplex. The groups introduced normally consist of conjugated hydrophobic moieties such as ethene, propyne, hexyne etc. alkynes provide the most stabilisation with propyne being optimum. The balance between increased conjugation and stearic interference must be considered when condensing such modification. It is known that modified part of the base only interacts with the base on 5`-side of the modified base within the strand, thus degree of stabilisation is sequence dependent. As the base can be modified in a stable fashion without interfearing with the hydrogen bonding. Acetylene group containing relative moieties are often added to allow labelling of bases e.g. fluorescent dyes via. Propargyl amino linker. The labelled bases are used in large number of molecular biology and medicinal assay.

1.3.(b). Sugar modification:

Sugar modifications have also been used to enhance stability and affinity. The α-anomer of 2`-deoxyribose sugar has the base inverted with respect to the natural β-anomer. ODNs containing α-anomer sugars are resistant to nuclease degradation and binds parllel to RNA target. The natural

2`-deoxyribose sugar is D isomer. An L-2`-deoxyribose analogue of cytidine (L-2`-dC) has been synthesised and incorporated in to ODNs[69]. The presence of L-2`-dC at the end of the ODNs enhanced the resistanced to exonuclease digestion but lowered the Tm with a complementry DNA. Modification of the 2`-OH of the ribose sugar form 2`-O-methyl or 2`-O-allyl sugar and is found to enhance resistance to degradation, compared with normal RNA, without compromising affinity[70]. Hexose sugars have also been substituted for the deoxyribose sugar and have been found to enhance stability but compromise affinity[71]. None of these sugar analogues are known to activate the Rnase H cleavage of the target RNA in an ODN:RNA hybrid, but they may prove useful when incorporated into ODNs targeted to regions of the RNA where a steric block mechanism is desirable.

1.3.(c).Modification of phosphodiester backbone:

Modification of phosphodiester backbone has been shown to impart stability and may allow for enhanced affinity and increased cellular permeation of ODNs. In addition many different strategies have been employed to replace the entire backbone with novel linkages. The chemistry required to produce these linkages is often quite challenging and many groups prefer to produce and characterise dimers containing the novel linkages, rather than synthesised fully substituted polymers. In this form they may be introduced during automated ODN synthesis, creating a polymer with alternating phosphodiester and novel linkage, allow biophysical characterisation. Normally backbone modifications are utilised to improve the stability of DNA to enzymatic degradation. When drugs consisting of DNA are used they are foreign pieces of DNA and as such are substrate for enzymes that control the amount of DNA within the cells. Unfortunately when backbone modifications are employed the stability normally decrease. Although this is not always the case.

(i). Phosphorothioate and Mthylphosphonate:

Modified ODNs have been attractive to biological researchers due to their availability through automated ODN synthesis. Both ODNs are stable to degradation by nucleases, but in general hybridisation to target sequence with lesser affinity then a phophodiester ODN[72]. The ODN`s containing either of these modification are a mixture of 2n diastereomers (where n is the number of linkage), and it is possible that an ODN containing all RP and SP isomer would hybridise with better affinity. Lesnikowski et al showed that for 7 nt oligothymidine with a methylphosphonate backbone, the all-RP ODN's containing all RP ODN's had a significantly higher Tm than the ODN which was mixture of diastereo isomer, while the all PS- ODN had Tm too low to determine[73].

Unfortunately chiral synthesis of these backbone analogues will require considerable technology development and does not seem likely in near future. It may not be necessary to produce chiraly pure ODNs for therapeutic use, but chirality still may introduce unwanted issue. For example,

batch to batch variation in the mixtures of isomers could affect activity and assuring consistent ratios of isomers will be difficult.

A phosphorodithioate version of the phosphorothioate has been synthesised and characterised. In this linkage both of the non-bridging oxygen have substituted by with sulfer. The linkage is highly nuclease resistant and achiral, the ODNs bind with slightly less affinity than phosphorothioate.

(ii).Carbonate and Carbamate

Carbonate and carbamate linkages have been introduced as a novel replacement for phosphodiester backbone (fig 1.7). The carbonate dimers are unstable to mild basic conditions and have been synthesised as dimers, but have not been incorporated into ODNs. The 5;-N-carbamate linkage is chemically stable and was synthesised as hexamer of cytidine (C-6) or Thymidine (T-6) . The C-6 carbamate ODN was found to bind a complementry DNA or RNA with high affinity, but the T-6 carbamate ODN bound with relatively low affinity. Stirchak et al introduced the morpholino 'sugar' which is inturn connected via a carbamate linkage (Fig.1.7) . Other N containing derivatives include the hydroxylamine linkage, methylene (methylimino) (MMI) and methyleneoxy (methylimino) (MOMI) linkages (Fig. 1.7) all these modifications have been introduced into ODNs and all shows slight increases in Tm with complementary RNA.

(iii) Peptide Nucleic acids

Recently a novel class of non-chiral, designed synthetic molecules- Peptide nucleic acids (PNA) (Fig 1.7), are emerged as potential antisense agent[74,75]. They were introduced by Neilsen et al. In 1991[76] as a replacement of the entire ribose- phosphodiester backbone (Fig 1.7). These have polyamide backbone similar to that found in proteins and nucleobase are linked at N through methyl carbonyl linker. PNA is chemically stable and in contrast to natural nucleic acid and peptides, PNA is expected to remain intact in living cells since it is not a substrate for natural hydrolytic enzymes and is not degraded in cell extract. PNAs have several properties that make its it promising for use as a gene-targeting agent.

PNA is capable of sequence specific recognition of both DNA and RNA by Watson and Crick base pairing and the hybrid complexes thus formed exhibit extraordinary high thermal stabilities. It has been that PNA invades DNA duplex in a sequence specific manner, displacing the existing DNA complementary strand and hence is ideal as therapeutic agent. PNA's have a strong binding affinity to DNA, even higher than complementary strand and hence may be ideal to target DNA within the nucleus and homopyrimidines PNAs forms triplex with PNA2: DNA composition[86].

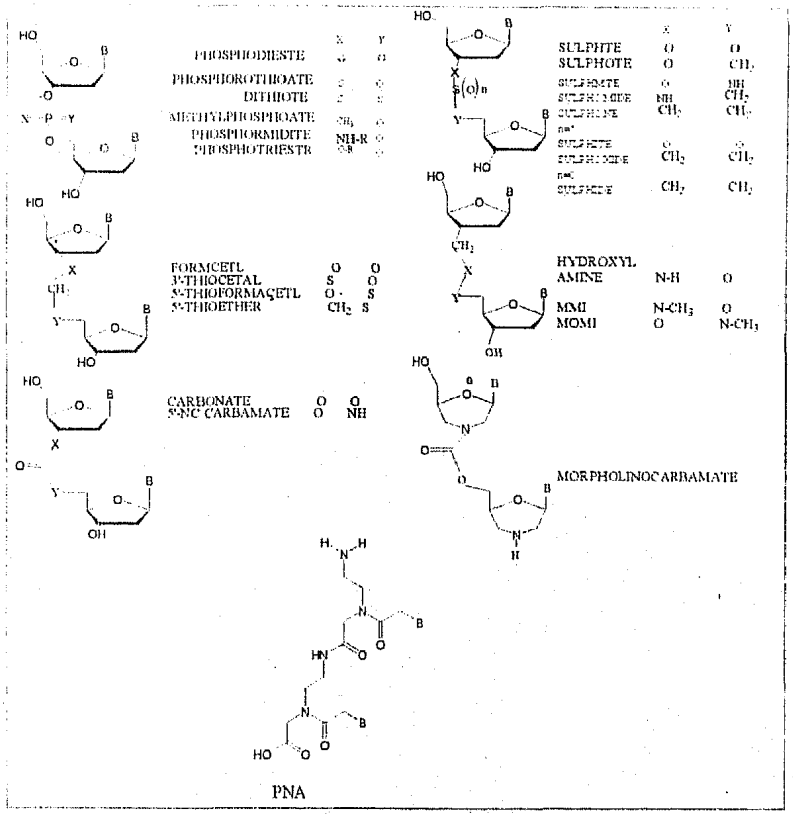

Fig. 1.7 Modification of phosphodiester backbone

However, PNA's have some drawback such as poor cell permeability and ambiguity in DNA recognition since these bind DNA in both parallel and anti-parallel modes. This has restricted their widespread application in biology although, they are being used as powerful tools as probes in Southern blotting and in application FISH. Summarised characteristics are as follows,

- PNA's obey Watson –Crick base pairing[87].
- Not recognised by nucleases and proteases[88].
- The Tm of PNA/DNA and PNA/RNA duplexes is not dependent of the ionic strength[89].
- Higher specificity of binding.
- Higher affinity for complementary DNA or RNA.
- Assembled routinely by peptide chemistry.
- Labelling and further modification possible[90].
- Some PNA constructs can invade dsDNA[91].
- FISH[92]
- PNA- directed PCR clamping, in which PNAs are used to identify point mutations in a PCR base assay[93].

1.4. Use of modified oligonucleotides in therapeutics:

During the past two decades, techniques based on the complementary hybridisation between oligonucleotides of known sequences (probes) and nucleic acid targets have been widely applied in genome sequencing, site specific mutations, molecular diagnostics, therapeutics and other allied

problems in Molecular Biology[94-99].There are mainly three oligonucleotide based therapeutic modalities, which are being used viz., antisense, antigene (triplex formation) and ribozymes[95-99].

1.4.(a). Antisense

The basic idea of the antisense approach is that once genetic make up of a virus is sequenced or the genes involved in the disease like cancer are identified, a synthetic strand of DNA can be designed that is a mirror image of the mRNA coded for by one or more of those genes. The antisense compounds binds to the mRNA like a piece of Velcro and interrupts the production of disease-associated proteins (The term antisense was used to describe this binding of opposites).

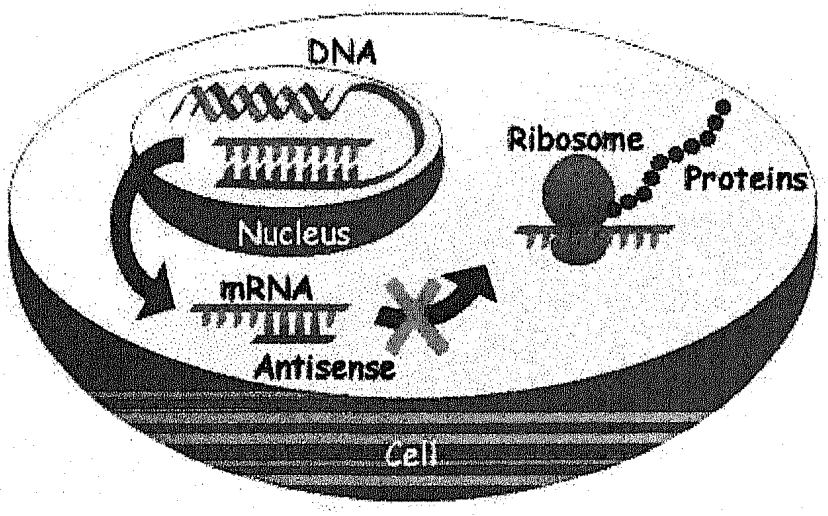

Fig. 1.8 Antisense methodology

The mRNA is the "sense" strand of genetic material while the synthesised complementary DNA is an "antisense" compound. In general, antisense refers to the use of small, synthetic oligonucleotides, resembling single stranded DNA to inhibit gene expression[100-102] by hybridisation. A major advantage of this strategy is in the potential specificity of action. In principle, an ODN can be designed to target any single gene within the entire human genome, particularly creating specific therapeutic for any disease in which the causative genes are known. As a result, there have been numerous studies of antisense ODN's activity for potential antiviral[103-105] anticancer application[106,107].

1.4.(b) Antisense RNA

Antisense was first discovered as a naturally occurring phenomenon in which cells transcribe an antisense RNA, complementary to a cellular Mrna[100,101,108]. This antisense RNA was found to be a repressor of gene expression, hybridising to a target mRNA, inhibiting its translation, and describing the cellular levels of the protein. Antisense RNAs inhibit gene expression through the activity of a cellular enzyme, which modifies double-stranded RNAs[109]. This enzyme recognises the RNA:RNA duplex, disrupts the base pairing and changes many of the adenosine residues to

inosine[110]. Gene expression is inhibited since the modified mRNA is no longer competent for translation.

1.4.(c) Antisense ODNs

In contrast to antisense RNA, no naturally occurring systems are known which can utilise antisense DNA to inhibit gene expression. Inhibition of gene expression through the use of exogenously added ODN (Antisense oligonucleotide) was first discovered by Zamecnick and Stephenson in 1978, to inhibit replication in Rous Sarcoma Virus by the addition of a phosphodiester ODN to tissue culture media, postulating that the inhibition was through an antisense mechanism[111].

Thus, the use of antisense oligonucleotide in therapeutic is well known since long back, but much progress could not be achieved due to the following reasons.

(i) Their intracellular instability (degradation by endonucleases)

(ii) Poor cellular uptake

(iii) Synthetic procedures for oligonucleotides were not well developed and

(iv) cost effectiveness.

However, later on great progress was made and number of synthetic procedures were developed. In recent years, considerable efforts have been made to the synthesis of chemically modified ODNs to improve their stability against nucleases, to enhance their binding affinity with the complementary target and to facilitate their uptake by cells[112-116]. In this context, phosphorothioates (PS) ODNs, in which one of the non-binding oxygen in an internucleotidic phosphodiester-linkage is replaced by sulphur have emerge as a promising first-generation antisense therapeutics.

These phosphorothioate oligonucleotides have been used to develop antisense-based drugs and their "carrier-mediated uptake" was improved by making various oligonucleotide conjugates[117-120]. During the past decade a number of such drugs have been developed and evaluated in clinical trials for certain disease like AIDS, rheumatoid arthritis and Cytomegalo Virus (CMV) infection and Crohn's disease. In 1998 Vitravene (Fomivirsen) first antisense based drug was approved by Food and Drug Administration USA for human consumption for the treatment of CMV infection[121,122]. Further use of antisense in functional genomics for drug treatment validation is also being suggested and demand for antisense-based drugs is increasing[122].

1.4. (d). Antigene

Triplex DNA first discovered by Felsenfeld *et al.*in 1957[123], has attracted considerable interest because of its possible biological function *in vivo* and its wide variety of potential applications such as regulation of gene expression, site-specific cleavage of DNA and mapping of genomic DNA[124] etc. In the antigene strategy the oligonucleotide is targetted to double helical DNA[125,126] and this is potentially useful for human therapeutics[127].

A triplex is usually formed through the sequence-specific interaction of a single-stranded homopurine or homopyrimidine triplex-forming oligonucleotide (TFO) with the major groove of homopurine - homopyrimidine stretch in duplex DNA. In the pyrimidine motif triplex, a homopyrimidine TFO binds parallel to the homopurine strand of the target duplex by Hoogsteen hydrogen bonding to form T.A:T and C^+. G:C triplets. On the other hand, in the purine motif triplex, a homopurine TFO binds antiparallal to the homopurine strand of the target duplex by reverse Hoogsteen H-bonding to form A.A:T (or T.A:T) and G.G:C triplets[124]. Increasing reports on inhibition of transcription, factor DNA interaction and the activity of restriction endonuclease (RE) by triplex DNA have tremendously implied its obvious possibilities for therapeutic application[128,129] and thus can prevent the onset of viral and genetic disease at much earlier stage vis-a-vis antisense (Fig 1.8).

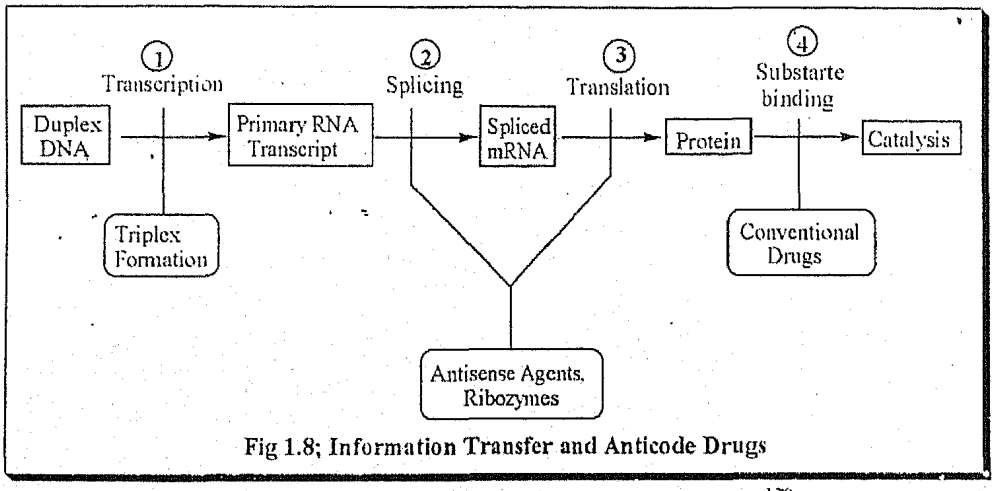

Fig 1.8; Information Transfer and Anticode Drugs

Some of the triple helix-based drugs being evaluated in clinical trials[130].

Company	Collaborator	Target	Status
Genta	Proctor and Gamble, Chugal Gen-Probe	HIV, Herpes Simplex Virus (HSV)	Phase II
Gilead Sciences	Glaxo	HIV	Phase I
Triplex Pharmaceuticals	Hoechst	HIV, HSV	Phase II

Table 1.1; Triple helix based drugs being evaluated in clinical trials

These are being used for variety of other purposes like viral target cleavage[131] and detecting DNA mutation[132].

1.4.(e). Ribozymes

Ribozymes are catalytic RNA's which, as part of mRNA structure, catalyse the self-splicing of the primary transcript[133-135]. These ribozymes posses the unique secondary (probably tertiary)

structures for cleavage activity and was first discovered by Cech[136] in 1981in *Tetrahymena thermo- phila*. Particularly of interest for therapeutic use six type of catalytic motifs have been reported so far viz. Group I introns[137], RNase P[138,139], Hairpin and Hammerhead[140,141], Hepatitis Delta Virus (HDV)[142] and *Neurospora* VS RNA[143]. The most advantageous aspect of ribozymes are that they can become active only after the hybridisation in comparison to artificial endonucleases.

By suitable chemical / molecular modifications, ribozyme can be designed to specifically base pair with and finally cleave any target RNA resulting in producing an irreversible inactivation of the target sequence which may be a viral genome. These not only bind to the target viral sequences but also cleave it. After the cleavage, ribozymes become free to process another target sequence. Thus, the ribozymes give multiple turnover unlike antisense and triplex constructs and consequently these catalytic RNAs are potentially far more effective as therapeutic agents. These may also be manipulated to ligate new pieces of RNA onto the target by trans splicing and thus may be used to create new gene function. Therefore, ribozymes can be used to destroy / modify any target RNA and in principle, can be used for any disease where a specific protein / virus can be linked to disease etiology[133-135]. These are currently being investigated for the treatment of cancer and AIDS[144,145]. Modern molecular biology faces extremely complicated experimental problems. Proteins, polysaccharides, nucleic acids, biological membranes and other ingredients of a cell interact, form sophisticated structures and accomplish numerous catalytic, regulatory and other functions. In the investigation of biological systems, one uses a broad arsenal of physical and chemical methods. The necessity of such an approach is caused by the specificity of biological systems. The study of biomolecules usually tends to address following problem e. g.,

(i) What is the precise structure at the atomic level of a macromolecule / or aggregate of macromolecules?

(ii) What properties of the macromolecule determine its structure and what forces participate in stabilising it?

(iii) If a macromolecule binds with other molecules, what is their structure, what is the number of binding sites and what is the intensity of binding expressed in the form of physical constants?

(iv) Where is a particular macromolecule located within the cell or a small unit like a virus?

Therefore, a researcher rather than seeking complete information, usually aims to derive the main structural and dynamic properties important in the functional activity of a system. Various methods based on colorimetry, electrophoresis, microscopy, immunology, hydrodynamics and spectroscopic properties are being used but of particular importance are biophysical labelling methods.

1.5. DNA Detection techniques:

Radioactivity, UV-spectrophotometery, Mass spectrometery, NMR, Crystallography, Vibrational-spectroscopy and CD are mainly used for DNA detection[146].but at present fluorescence[147] is widely used for DNA detection. (Table 1.2)

NAME	ADVANTAGES	DISADVANTAGES
Radioactivity	Highly sensitive ($<10^{-12}$M) and provides a quantitative signal allowing accurate determination of the amount of DNA	Requires special caring in handling short half life, leading to restriction in application
UV-Spectrometery	The average λ 240-280nm, thus can be used to detect DNA	Sensitivity is not very high with the cut off of concentration for DNA being around 10^{-7}. No sequence differentiation is possible.
Mass-Spectometery	Allows the mass of DNA to be determined extremely accurately normal by either elsetrospray or MALDI time of flight methodologies.	Need for expensive equipment. Treat ment of sample is also necessary prior to ionisation and is difficult.
NMR Spectroscopy	The signal obtained depends environment of H & as such give a very accurate picture of DNA.	Large amount of DNA is require to asses and must be of higher degree of purity.
Vibrational-Spectroscopy	Change in structure due to interaction with other molecule can be monitored.	Disadvantage is sensitivity. No information of sequence composition.
Circular -Dichroism	Relies on difference in adsorption of polarised light by helics with different orientations i.e. left or right handed.	Spectra is hard to detect.
Crystallography	Use of X-ray crystallography has played a major role in understanding of DNA structure and function.	Main disadvantage in using crystallography with DNA is needs to grow a crystal with good diffraction properties. This can be very difficult.
Fluorescence	Defined as the emission of radiation as a molecule return to its ground state from an excited electronic state. Fluorescence is highly sensitive (10^{-12}M).	If labeling of strand is required then chemical modification is necessary, as is separation of the strand prior to analysis due to the overlapping nature of fluorescence emission profile.

Table. 1.2 Physical DNA Detection Techniques.

1.5. (a).Modern techniques for DNA analysis that use fluorescence:

Fluorescence is defined as the emission of radiation as molecule returns to its ground state from exited electronic state. Natural DNA produces very weak fluorescence[147] at room temperature and as such additional molecules, which exhibit strong fluorescence, known as fluorophore are used. These fluorophore attached to DNA by chemical means or designed to fit between the base pairs in duplexes. Fluorophore that fits between base pairs in a duplex are known as intercalators and only fluoresce when intercalated thus providing a method distinguishing ds and ss DNA. Fluorescence is also quantitative thus can be used to measure the amount of DNA.

Many methods exist that employ fluorescence for DNA detection and analysis. The most recent technique to emerge provide information on specific sequence and their composition. In other words it is not possible to find a single base mutation, identify it and also provide a measure of quantitation. The following three examples are the best of current methods and all makes use of allel specific PCR perhaps the biggest advantage of these methods over any other is the homogenous nature of the assay i.e. separation of unincorporated primer is not necessary.

1.5.(b).TAQMAN assay:

Method of measuring the amount of a specific sequence presents by fluorescence. The basic principle involves using a probe that does not emit fluorescence until the incorporation of by an enzyme such as polymerase[148]. Hence unincorporated probe does not interfere with the procedure and makes use of the enzymatic specificity in a similer way to PCR. Made possible by use chemically modified primer sequence. The primer sequence contains a 5`-fluorophore e.g. FAM and a 3`- fluorescence quencher e.g. TAMARA. Thus the probe does not display any fluorescence yet hybridises to the target sequence of the gene under scrutiny. A downstream primer is also used. The normal primer starts the PCR and generate ds DNA. The PCR continues until it hits the double stranded produced by hybridisation of labelled probe. Displacement occurs and if then is exact sequence hybridisation the 5`- nuclease activity of the amplilaq gold enzymes is used digest the probe strand. This liberates the label and moves it away from the quencher to allow fluorescence measurements to be taken.

1.5.(b).Molecular beacons:

The strategy makes use of a hairpin loop in the labelled probe to bring a quencher into close proximity and quench fluorescence emmission[149]. Again the probe is not the part of the primer used in PCR and allele specific primer must still be used by varying the temperature of the mixture the probe will bind to the target sequence and break the hairpin loop allowing the fluorophore to emit and allow quantitative measurements to be obtained. The probe sequence is designed to be complementry to part of the amplified product and different fluorophores can be used, as only one colour will be emitted. It is not as accurate as 'TAQMAN' as not the part of the growing sequence.

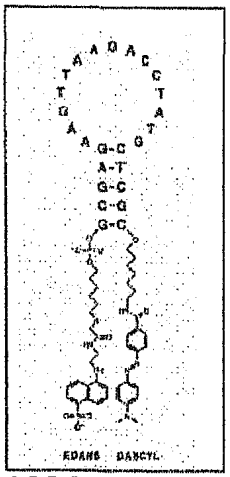

Fig. 1.9 Molecular beacon

1.5.(c).Fluorescence resonance energy transfer(FRET)

FRET is an example of fluorescence related phenomenon[150] where fluorescence spectrum of one fluorochrome, the donor overlaps with the exitation spectrum of another fluorochrome, the acceptor and when the donor and acceptor are at close proximity (>70A^0), exitation of the donor induces emmission of fluorescence from acceptor by dipole induces emmission of fluorescence from acceptor. The process involves transfer of excited state energy from donor to acceptor by dipole-induced dipole interactions[151].

FRET is now being used in nucleic acid base biomolecular investigations to detect oligonucleotide hybridisation (DNA-DNA, DNA-RNA, RNA-RNA, RNA-PNA, PNA-PNA), intracellular stability of oligonucleotides, ligation and self cleaving activity of ribozyme, triplex formation and nucleic acid sequencing[152]. The wide range of application of FRET in nucleic acid and oligonucleotide chemistry has recently been reviewed[153].

FRET offers an increased advantage over other techniques because

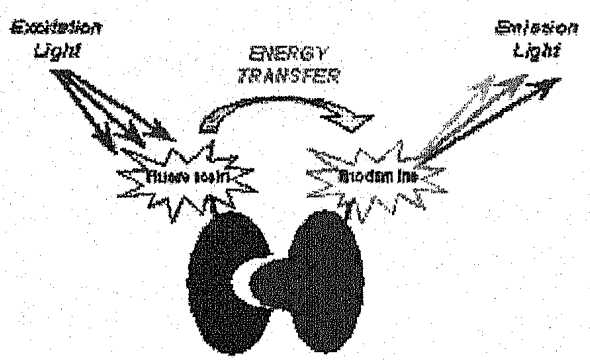

Fig. 1.10 FRET energy transfer

- The donor acceptor energy transfer considerably improves the sensitivity of detection.
- The real time monitoring of various processes is possible without adhering to any separation or standard workup procedure

FRET is being used in a big way to investigate various processes associated with nucleic acids

Present Work

The work deals basically with designing, synthesis, and characterisation of PNAs as DNA mimics. The synthesised PNA monomer was coupled with synthetic sequence of DNA at one terminus, this PNA monomer was labelled with rhodamine. Its complementary sequence was labelled with fluorescein, to be used as probe. Extent of hybridisation of rhodamine labelled PNA monomer tagged synthetic DNA with fluorescein labelled 7-mer was studied and assessment of binding was further confirmed with FRET.

The work has been described under five chapters. Chapter one is introductory and gives detailed account of synthesis of oligonucleotides, types of modifications carried out inclusive of PNA (Peptide nucleic acid) and DNA detection techniques with special reference to fluorescence

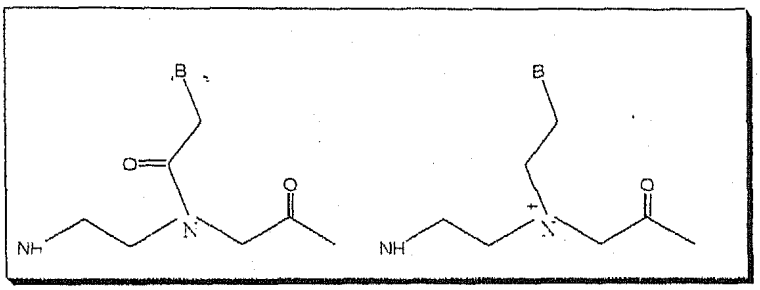

Fig. 1.11 Conventional PNA monomer along with our modified monomer eth B (on right)

measurements. Chapter two describe in detail the designing and characterisation of PNA monomer (N-(t-Butoxycarbonylamino ethyl)-N(6-N-benzoyl, 9-ethyl)glycine).

It also deals with types of modifications in PNA at base, linker and backbone level in order to improve its stability and bioavailability. Various approaches adopted and types of protecting groups used are also described. Chapter three gives details of the synthesis and labelling of two heptamer sequences

5`-*flu*-AAT GGA T-3` (*flu* = fluorescein, labelled with fluorescein) and

5`-*rh*-A*TC CAT T-3` (*rhA**=Peptide monomer labelled with rhodamine) via. Phosphoramidite approach and its coupling with rhodamine labelled PNA monomer. Labelling of its complementary heptamer with fluorescein was also carried out at 5`- terminus. The chapter also mention about different labelling methods for DNA and PNA`s and their use as probes.

Chapter four deals with the details of hybridisation studies of fluorescently labelled sequences 5`-*flu*-AAT GGA T-3` (*flu* = fluorescein,) and 5`-*rh*-A*TC CAT T-3` (*rh*= rhodamine, A* is modified PNA monomer). In this chapter hybridisation is studied by melting temperature (Tm) . The effect of environment on PNA hybridisation with reference to strand invasion and triplex formation has also been discussed in this chapter.

Finally, the last chapter five gives a clear view of FRET studies carried on with fluorescently labelled sequences 5`-*flu*-AAT GGA T-3` (*flu* = fluorescein, labelled with fluorescein) and

5`-*rh*-A*TC CAT T-3` , here fluorescein is a donor dye while rhodamine acts as an acceptor.

In short the main objective of this study is to assess the effect of introducing a peptide monomer in a DNA sequence, which has been studied by melting behaviour and also by FRET. This study is of great significance in DNA mimicking

Note: A denotes a PNA monomer containing adenine nucleobase.*

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CHAPTER-2

Synthesis and Characterisation of a monomeric unit of Peptide Nucleic Acid (PNA)

2.1 Introduction:

Short synthetic oligonucleotides, as highly specific inhibitors of translation and transcription have shown great promise as antisense and antigene therapeutic drugs, specially during the last one decade.

The quest for oligo analogues and mimics with improved properties in terms of nuclease resistance and target affinity have led to the development of a large number of DNA analogues. Recently, the properties of a novel type of DNA mimic Peptide Nucleic Acids (PNA's), has led us to modify our view of the uniqueness of DNA. Peptide Nucleic Acids (PNA), invented approximately ten years ago, are emerging rapidly as useful molecules in DNA hybridisation and antisense technique. Although therapeutic drugs based on PNA's are still not in sight, applications in diagnostics, in particular in situ hybridisation and PCR based systems, are making rapid strides. The simple structure of PNA in combination with impressive DNA mimicking property has already provided inspiration to many synthetic organic chemists for preparing several derivatives and analogues towards improving their physio-chemical and biological properties.

Peptide Nucleic acids (PNA's) have been around for more than ten years and during this time some, but not surely all of the promises expected from this molecule have materialized. Major success has been achieved within the diagnostic use of PNA oligomers in hybridisation (in particular in situ hybridisation) and PCR based technologies[1-3].

The development of PNA oligomer into gene therapeutic drugs is still in infancy. However major progress in particular concerning their cellular delivery has been made within the past couple of years[4,5] . However, the DNA mimicking properties of PNA's coupled with their simple structure and easy have led to the synthesis of a large number of analogues. The aim being to prepare analogues isosteric with natural DNA's e.g. the analogues of aminoethyl glycine PNA's which can give stable duplexes.

2.1.1 PNA and origin of life:

PNA like molecules have been proposed as candidate for a prebiotic genetic material preuding RNA and DNA [6]. Nucleobases and amino acids are quite easily obtained under conditions thought to imitate the primitive earth as elegantly demonstrated by Miller[7] and Oro[8], in 1950's where as it is extremely more difficult to obtain ribose and nucleobases under such conditions. Indeed, Miller and coworkers recently showed that PNA precursors are possible prebiotic products[9]. Furthermore, information transfer by chemical oligomerisation between PNA and DNA is also possible, albeit with low efficiency[10]. Therefore, Peptide nucleic acids like polymer should be considered in the discussion of a genetic material of prebiotic life.

2.2 Chemical modifications of PNA:

The structure of the classical PNA monomer *1* has been subjected to a variety of rational modifications with the aim of understanding the structure activity relations in the class of DNA mimics as well as obtaining the PNA oligomer with specifically improved properties for various applications within the diagnostics, medicine, molecular biology etc. these limitations include low aq. Solubility, ambiguity in DNA binding orientation and poor membrane permeability, structurally the analogues can be derived from modifications in the ethylene diamine or glycine sector of the monomer, linker to the nucleobase, itself or a combination of the above. The strategic rationale behind the modifications are,

1. Impart rigidity to the PNA backbone via. Conformational constraints to favour duplex formation entropically.
2. Introduction of cationic functional chirality in a PNA backbone, in a side chain substitution.
3. Modulate nucleobase pairing either by modification of linker or the nucleobase itself and.
4. Conjugation with transfer molecules for effective penetrartion in to the cells.

I. Backbone modification:

Several modifications have been introduced to the PNA backbone with a view in mind for application in diagnostics. The earliest and the simplest of the modification involved extension of the PNA structure with a methylene group in each of the structural sub-units, aminoethy[32], glycine[33], and base linker[33] of the PNA monomer. These resulted in PNA's with N-(2-aminoethyl)-β-alanine *2* and N-(3-aminopropyl) glycine *3* backbone and ethylene carbonyl linked nucleobases *4* however, these modifications resulted in significant lowering of Tm. The replacement of the tertiary amide carbonyl by a methylene group leading in a flexible, cationic tertiary amine monomer *5* resulted in a destablisation of the PNA:DNA hybrids[34]. The necessity of such pseudo rigid amide groups pointed to the importance of constrained flexibility in the backbone.

Further rigidification of the PNA backbone has been attempted by the introduction of the alkyl substitution individually or simultaneously in the aminoethyl *7a* or glycine [35-38] segment or in both. Cyclic structures may be generated with 1,2-cyclohexylamino *8* and spirocyclohexyl *9* rings in monomer[39,40].

While PNA that having (s,s) cyclohexyl, ring in the aminoethyl part hybridise with complementary DNA similar to unmodified PNA , those derived from (R,R) cyclohexyl moiety *8* lacked such property[41]. Thermodynamic data showed that DNA binding of SS- isomer PNA accompanied by a reduced loss in entropy, but this was counter balanced by a decreased gain in enthalpy[41].Glycine may also be substituted by other amino acids leading to chiral PNA *7*(R1= H) i.e hydrophobic, hydrophilic or charged substituents[42]. PNA oligomers having chiral monomers have the same

hybridisation properties. Substitutions with L/D- alanine showed a slight preferences for antiparllel binding with DNA, with D-alanine being slight better than L-alanine[43]. If the D-gluconic acid and D-Lysine are the substitution as chiral monomers the sequence selectivity of PNA oligomers increases. With D-lysine there is the added advantage of solubility in aq. Systems.

Another type of modification involved interchange of various Co and NH groups on the peptide linkage leading to retero- inverse[44] *10*, peptoid[45] *11* and heterodimeric[46] *12* analogues. In all these systems interbases residue separations are similar to the unmodified PNA, but accompanied by inversion of intra and inter residue amide bonds. They exhibit lower potency for duplex formation with complementary DNA / RNA suggesting that in addition to geometric factors, other important requirement such as hydration and dipole-dipole interaction etc. influencing the micro environment of the backbone may be involved in effecting PNA:DNA hybridisation. But the heterodimer analogues exhibit the same potency for hybridisation.

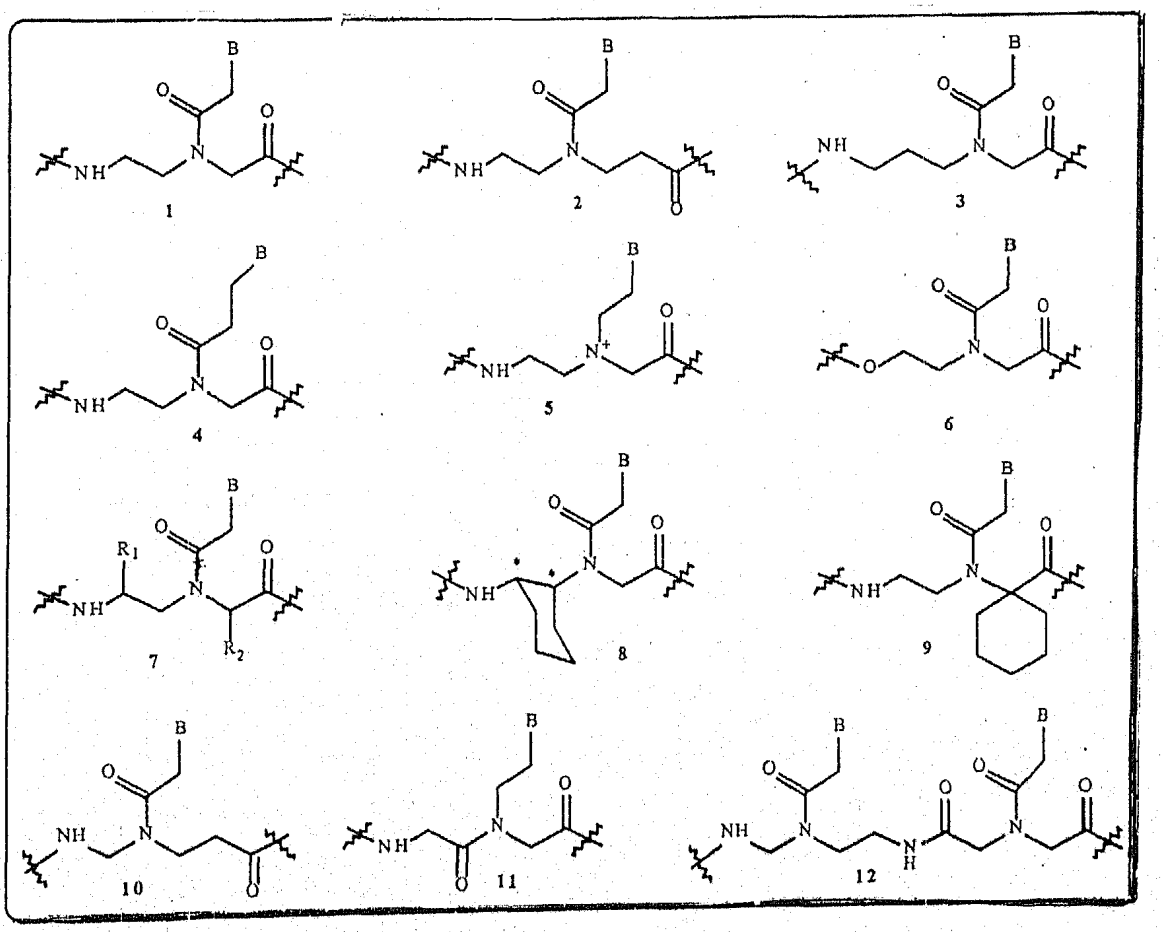


Fig. 2.1. PNA monomer modification *1-12*

Modified PNA monomer may also be siynthesised by using α-amino acids carrying nucleobases building blocks (enantiospecific nucleo amino acids). The nucleobase are attached either on the side chain[47] or appropriately functionalised at the α-carbon site[48] . In the latter case the original side chain of the amino acid is involved in the making of PNA backbone.

II. Modification of linker

the monomer of type *13* having a long linker than the usual one, showed no significant interaction with polynucleotide [48]. Recent studies on duplex formation from oligomers analogues of *13* and *14* with DNA have not been encouraging [49]. However, the corresponding adinylic analogues interspaced with glycine, serine, threonine or tyrosine exhibited strong affinity with poly dT and poly U to form triplexes. Heterodimeric PNA`s *15 -19* derived from alternating monomer of type *13* α-amino acids have been reported[50 (a,b)] but did not showed any improved hybridising property PNA`s composed of monomer *20* derived from serine and homoserine coupled by ether linkage with glycine or alanine were able to bind sequence specifically to RNA though with much weaker affinity[51,52].

An important advantage exhibited by such type of ether linkage in the side chain is the all or none type hybridisation, useful for the detection of even single mismatched DNA. indicating a very high sequence specificity[52]. Nucleic acids analogues composed of glycine and nucleobase linked β-amino alanine *19* (X=NHCO) are repeated but without any data on stability on their derived hybrids. Novel glucopyranosyl peptide nucleic acids (GNA) based on sugar nucleoamino acid monomer *21* derived from configurationally locked, conformationally constrained sugar have been synthesised[53]. These binds with RNA with overall affinity equivalent to that of DNA:RNA hybridisation, but with a better sequence descrimination ability. Thermodynamic parameters have been suggested an entropic gain in GNA due to pre-organised scaffold[53]. An aromatic moiety was introduced in PNA backbone by employing monomers derived from (S)-2-hydroxy-4-(2-aminophenyl) butanoic acid *22*, carrying a nucleobase and preliminary studies on a tetramer suggested favourable base stacking interactions[54]. In other PNA modification the central amide link between base and backbone with somewhat restricted rotation was replaced by a geometrically restricted function such as double bond[55] *23*. Such olifinic PNA`s bound with complementary DNA with a reduced affinity and interestingly, even homopyrimidine sequence formed only duplex with preferred parallel mode of modified PNA`s the result emphasize the importance of electrostatic effects of the amide bonds in influencing the micro-environment for favorable interaction of subunits.

PNA`s, in which inter-residue amide bonds was replaced by phosphonic acid[56 (a,b)] *24* phosphoinamide[57 (a,b,c)] *25* or phosphoramide[58] *26* linkage have shown interesting DNA duplexation

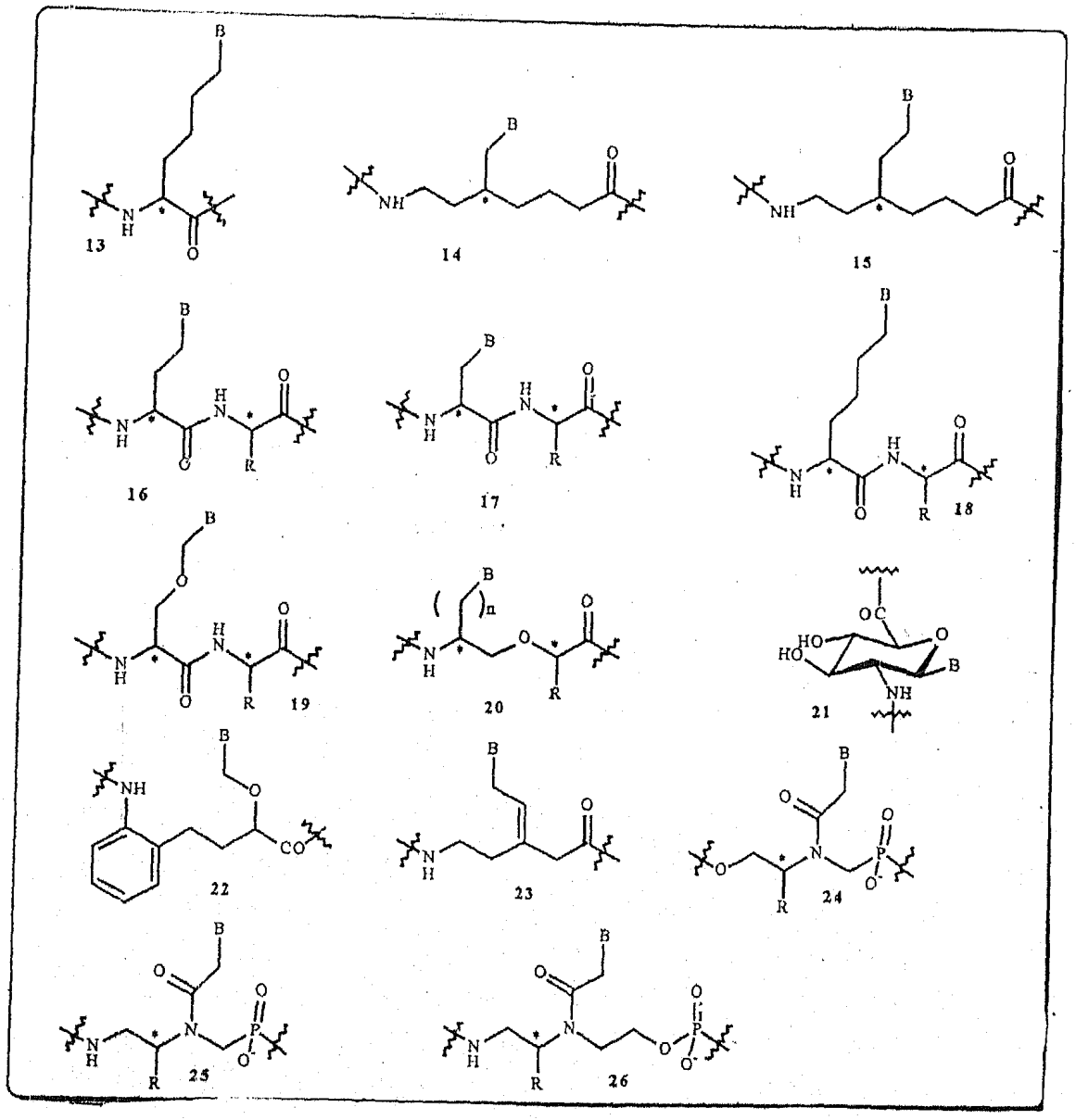


Fig. 2.2. PNA monomer modification *13-26*

properties. A number of structural variations in a combinatorial sense in these analogues have been explored and all form viable complexes with complementary DNA as well as RNA, though with slightly lower Tms[56-59]. The chiral version these analogues are similar to original PNAs, exhibited no preferential orientation to DNA[60].

The negative charge of phosphonic PNAs led to excelled aq solibilities properties. The chimeric oligomers derived from alternating monomer of PNA and phosponic PNA forn duplex with DNA and may have promising properties.

III. Introduction of cationic functional chirality in a PNA backbone,

Recently, a novel class of cationic PNA (DNG/PNA) anlogues *27* have been reported [61] These are chimeric PNAs made of alternating monomers of PNA and DNG in which the O-(PO)2-O linkage of a nucleotides is replaced by stongly cationic guanidino (N-C (-NH)-N) funtion. These analogues a back -bone compose have mixed positive and neutral linkers. Bind DNA/RNA target with high affinity. The association is favored due to electrostatic attraction of the notable lack of hysteresis in melting /cooling curves suggested the cause of stability due to increased rate constant for association. The (DNG-PNA)2-DNA triplex, but higher Tm as compared to (PNA)2 –DNA triplex , but higher Tm then the corresponding (DNA)2-DNA triplex, with each mismatch causing a lowering of Tm by 13o which is comparable to that seen in PNA. PNA with inter residue linkage involving thiourea *28* have also been recently reported [61]

One of the relatively successful modification so for the PNA derived from monomers based on 4- [62, 63] *29* The two asymmetric center on the praline core at C2 and C4 lead to four diasteromeric monomer and one can expect different hybridization properties of PNAs composed from such chiral monomers, synthetically al these can be obtained from L-4 –Amino proline which is abundantly available, 4-amino proline nuclease also offers the felxibility to increase chances the position of the nucleobase of back bone among the different nitrogens, leading to oligomeric PNA Prolye peptide nucleic acids 30 based on partial substitution of L-4 –trans 9-aminopropyle units exihibited tendency to hybridise nucleic acids similar to that of unmodified PNAs [64,65] . . In sharp contrast, analogues PNAs derived from L-4-cis –amino prppyl or d-4 trance-aminopropyl units in backbone, significantly decreased the DNA binding properties as compared to achiral PNA . However, interestingly and significantly decreased. inclusion of even a single 4-amino propline into a PNA sequence, either at N-terminus or in the interior. Not only led to stabilisation of derived PNA –DNA hybrides but also effected significant discrimination in the orientation of binding. The chirality of the incorporated 4-amino proline seems to influence the stability of such PNA-DNA duplex decreased with increasing numbers of chiral prolyl substitution and the four homo oligomers derived from each of the diastereomers completely failed to form duplex [66] .

Negative results were obtained with PNA derived from 4-nucleobases substituted prolyl glycyl amide monomeric units [67(a,b)] *31*. Unlike other PNA`s including the 4-aminopropyl analogues *30*. this has a tertiary amide group with the amide nitrogen part of a cyclic ring system on the backbone. This leads to highly rigid structures that are not poised for effective duplex formation. The replacement of the tertiary amide carbonyl on the backbone by a methylene group relieves such a strain to generate N-(2-aminoethyl) propyl PNA`s [68] *32*. These showed remarkable biophysical properties in terms of PNA2-DNA triplex stabilities. Hitherto unprecedented higher melting of the derived PNA"DNA hybrids, reflected very significantly enhanced DNA affinity,

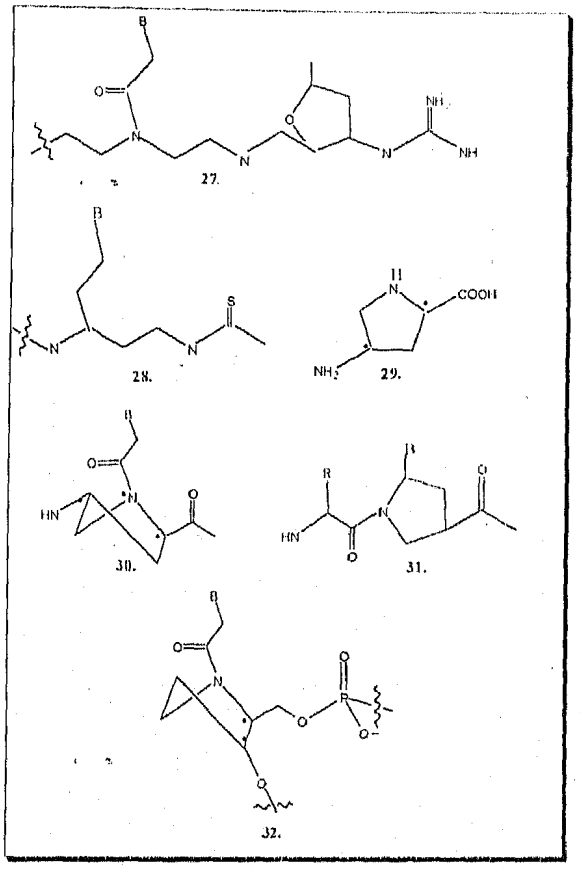


Fig. 2.3 Synthesis of modified PNA monomer *27-32*

while retaining the base pair discriminating power. The aq. Solubility of aep PNA's were at least 25-50 times more better than unmodified PNA's to date and makes this analogues of prime interest for further exploration.

A notable modified related related to the propyl nucleus is seen in oligonucleotides in which the sugar is replaced with stereoisomer of 4-hydroxy- *33* and 3-hydroxy-*34* N-acytyl prolino. These oligo's form stable complexes with complementary sequences. However, these are more a kind of DNA analogue than to DNA analogues[69].

IV. Nucleobase modification

there is increasing interest in modulating and expending the recognition motifs of standard base pairs.

employing non-natural nucleobases ligand in place of natural nucleobases would help to understand the recognition process in terms of various factors contributing to the event such as hydrogen bonding and nucleobase stacking. Further, new recognition motifs may also have potential application in diagnostics and nanomaterial chemistry. This when coupled with high affinity and strand invasion property offered by PNA would add a new dimension to PNA application. The non-stranded nucleobases employed so far with PNA are limited, compared to

repertoire of backbone modification desired earlier. 2,6-Diamino purine[70] offers increased affinity and selectivity for thymine and pseudoisocytosine[71] *36* very sufficient mimic of protonated cytosine for triplex formation. 2-amino purine [72] *37* hydrogen bonds with U and T in reverse Watson Crick mode and has the advantage of being inherently fluorescent to enable study of kinetic events associated in hybridisation. The E-base[73] was rationally designed for recognition of T"A base pairs in the major groove and form a stable triad with T in central position. The hypoxanthine base[73] linked to standard PNA binds A most strongly when T is its neighbor and binds C, when A is its neighbor and can thus effectively replace T or G under above circumstances. 2-Thiouracil *40* in a PNA chain opposite to 2-6-amono purine *35* in DNA has been effectively used as sterically compromised base pairs in a double duplex invasion in PNA complex[16] N^4-Benzoylcytosine *41* present in PNA chain has has been shown to cause inhibition of triplex formation[74] . another base introduced into PNA oligomer is 6-thioguanosine *42* which effected a characteristic shift in wavelength of absorbance due to hybridisation, but decreased the PNA"DNA hybrid stability[75].

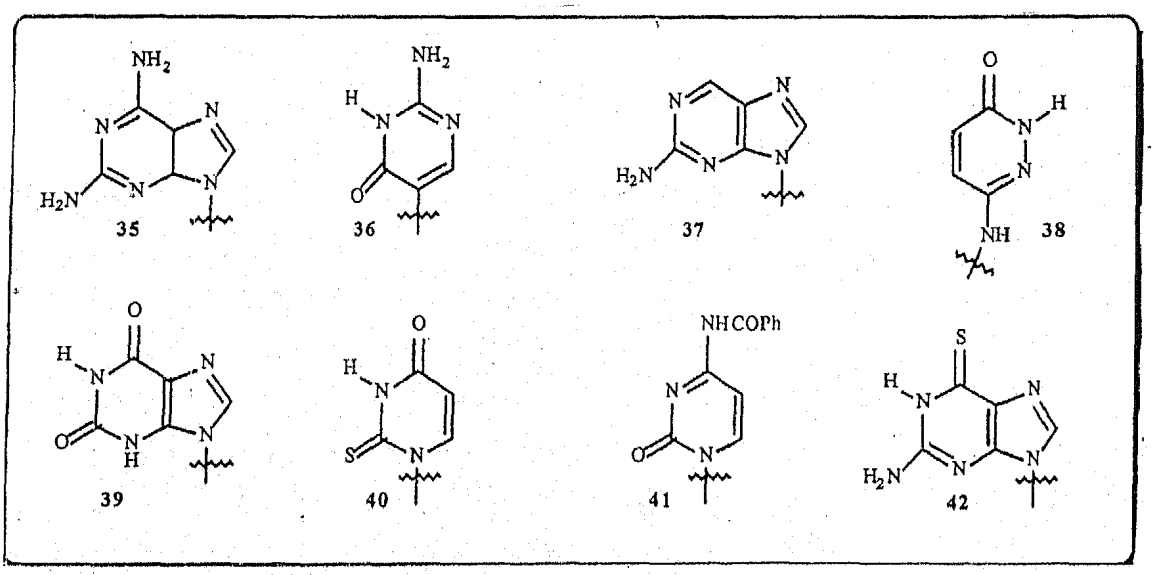


Fig. 2.3 Nucleobase modification

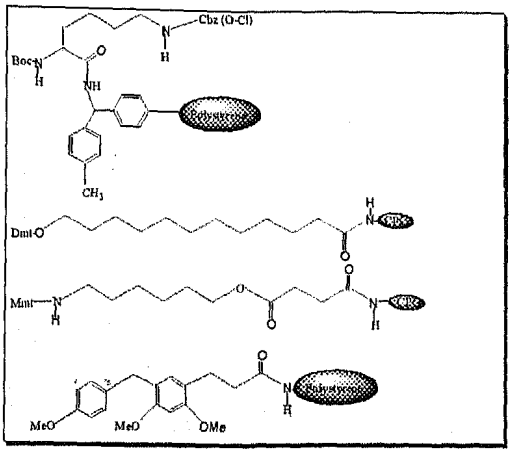


Fig. 2.5 Different solid supports used for PNA and PNA/DNA chimeras

1. Boc-Lys (Cl-Cbz)-MBHA-Polystyrene, 2. Dimethoxytrityl-12-hydroxydodecanoic acid-CPG, 3. Monomethoxytrityl aminohexysuccinate, 4. Breipohl linker,

2.3 Protecting groups used in PNA synthesis:

1. Monomers with acid labile protecting groups

the first reported synthesis of PNA[76] used monomer with the trifluroacetic acid labile BOC group for N-terminal temporary protection [76-79] . The exocyclic function group of nucleobase are protected with benzyl groups, which can cleaved with liquid hydrofluoric acid or triflurомethane sulfonic acid. The synthesis of PNA monomer with this protecting group combination and the building blocks described above is shown in fig.

Standard peptide coupling reagents, such as, DCC/HOBT or PyBroP, are used to attach the nucleobase acetic acid derivatives to the sec. Amino function of BOC-protected glycine ester. These BOC monomers are unstable for synthesis of PNA / DNA chimeras, as the strongly acidic condition (TFA/HF) necessary of DNA part of oligomers. The Mmt protected monomers described by Neilsen and other[80,81] can be deprotected under mild condition (TCA) and thus do not have this advantage. In particular the combination with base-labile protecting group for the exocyclic amino functionality of nucleobase cytosine, adenine and guanine allows deprotection condition that are compatible with standard oligonucleotide synthesis. Protecting groups established in oligo synthesis such as the benzoyl, anisoyl and ter-butyl benzoyl group for cytosine, adenine or isobutyryl and acetyl group for guanine are especially suitable in this context.

2.Base labile protecting groups

So far only Aeg monomer incorporating the base labile Fmoc protecting group have been described .

The synthessis of these monomer building block is shown in fig. A combination of Fmoc with Mmt group which can be cleaved under milder condition [82]

- **The ter-butylcarbonyl/ Benzyloxy carbonyl (Boc/ Cbz) protecting group strategy**

The earliest PNA to be synthesized were homopolymer of thymine[83] . They were prepared by Merrifield solid phase synthesis on Bc-L-Lysine amide derivatized MBHA polystyrene resin fig. 2.5 and they thus obtained as C-terminal lysine amide. The positive charge of lysine side chain help to suppress the undesirable tendency of PNA's to self aggregate and simplifies their purification and characterization.

The use of monomeric building blocks based on Boc protected thymine, (N^4-benzyloxy carbonyl) cytosine [84] , (N^6-benzyloxycarbonyl) adenine and (O^6-benzyl) guanine[85] in combination with in situ activation by DCC in 50% DMF/DCM was reported to give coupling efficiencies of 98-100% per cycle. In 1994 the first detailed description of the preparation of monomer and their use in PNA synthesis was published[86]

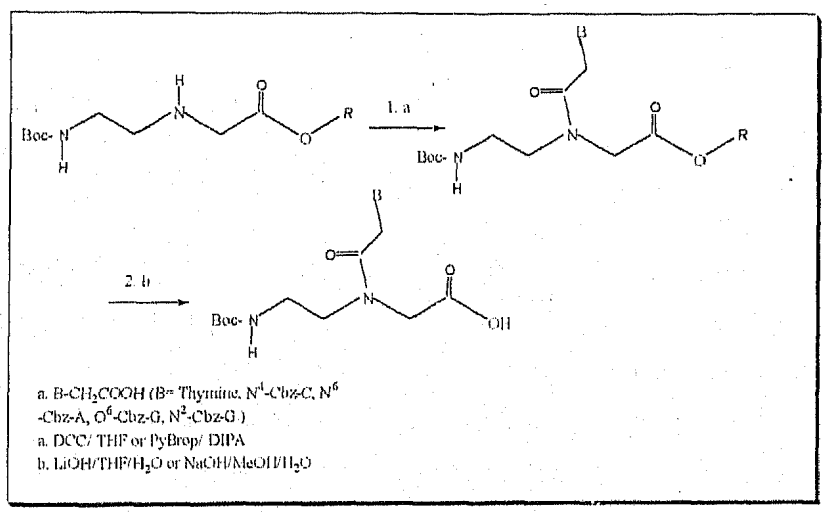


Fig. 2.6 The ter-butylcarbonyl (Boc) protecting group strategy-

Cleavage of the N-terminal Boc group was carried out with 50% TFA in DCM and coupling, any unreacted amino functionalities were capped with acitic anhydride in pyridine / DCM. Cleavage of these mixed sequence PNA oligomers from the solid support was carried out with Hf 2.55 thioanisole and the products were purified by reverse phase HPLC.

The Boc /Cbz protected PNA monomers can also be used I conjugation with HATU activation for the synthesis of PNA's on a polyethylene glycol-poly-styrene graft polymer[87] .

- **Fmoc strategy:**

PNA monomer that utilise the Fmoc group for the temporary protection of benzyloxycarbonyl nucleobases have been synthesise and used for the PNA oligomer synthesis[88]. PNA's were

obtained as their C-terminal amide or C-terminal acids when MBHA/MBHA-pik or chloro trityl polystyrene support were used respectively for both the synthesis. Cleavage of N-terminal Fmoc group was carried out with piperidie in DMSO/nmp. On the completion of oligomer synthesis the final N-terminal Fmoc group was retained and the PNA was cleaved from the support with HF/Anisole. The intact terminal Fmoc group could be used as a lipophilic handle to facilitate separation of the full length PNA. From failure sequence by reverse phase hplc and could be removed after purification by treatment with piperidie. However, cleavage of -terminal PNA unit was observed as a new side reaction on a longer treatment with piperidine. On the other the N-acyl transfer reaction that had been reported previously[89] was not detected under these condition.

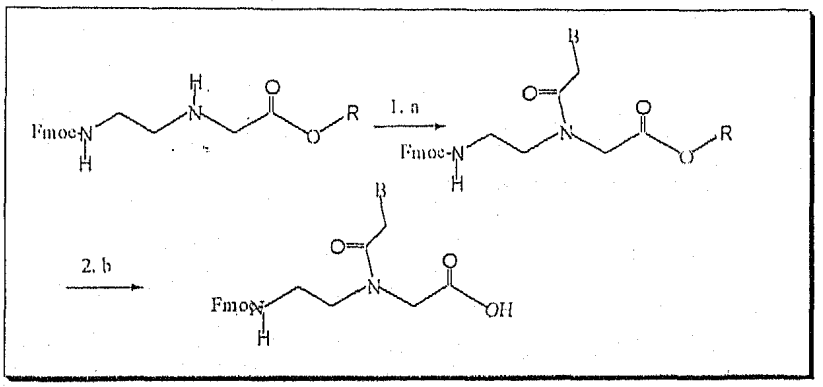


Fig. 2.7 Fmoc strategy

A further variation from the Fmoc Protecting group synthesis strategy has been described by Bergman et al[90]. an Fmoc /acyl protecting group strategy was prepared for the 4-bezoyl cytosine, 6-benzoyl adenine and N2- iso butyryl guanosine derivatives monomeric building blocks. Coupling was carried out with HATU/DIEA in DMF and capping with acitic anhydride in DMF. The use of a 12-hydroxydodecanoic acid derivatized aminoalkyl- CPG support allowed the attachment of the first PNA unit through a ester group. The synthesized oligo was cleaved together with the acyl protecting group of the nucleobases, by treatment with a solution of anhydrous ethnolic ammonia.

- **The 4-methoxypheyldiphenyl methyl/acyl (Mmt/acyl) protecting group strategy**

In a effort to find a milder method for pa synthesis that would be compatible with the condition for DNA synthesis, developed a new strategy that use the Mmt group for the temporary protection of the N-terminus and acyl protecting group for nucleobases[91].

The lipophilic Mmt group improves thee solubility of monomers in polar organic solvents, such as DMF or acetonitrile. The solubility of monomers could be further improved by the use of ter-butylbezoyl or anisoyl groups instead of the more commonly used unsubstituted benzoyl protecting group. The synthesis cycle is shown in fig. 2.8

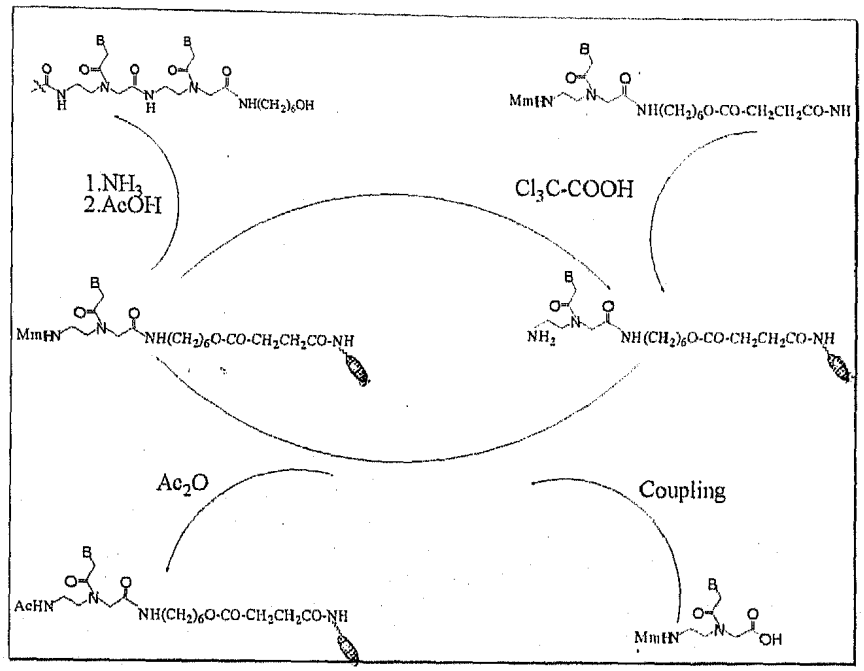


Fig. 2.8 PNA synthesis cycle for Mmt/Acyl-protecting group strategy.

Favored PNA coupling agents are HATU, and PyBOP.

The temporary protecting group strategy Mmt can be cleaved under very milder condition (3%trichloroacitic acid in DCM) and the coupling efficiency can be determined very easily by measurement of the colored Mmt cations released. The coupling step itself was carried in DMF by using a short preactivation with HATU or PyBOP in the presence of DIEA. Capping in an analogous manner to DNA synthesis was carried out with acetic anhydride/ lutidine/N-methylimidazole in THF.

2.5 Present work

The deoxyribose phosphate backbone of Oligodinucleotides has been extensively modified in an attempt to increased in vivo stability and to promote cellular permeation. In the recently reported peptide nucleic acids the deoxyribose phosphate backbone of an Oligodinucleotides has been replaced by a (2-aminoethyglycine unit). The synthesis of peptide is more versatile than oligonucleotide synthesis, allowing the facile design of a achiral backbone and large scale production. We therefore designed peptide nucleic acids (PNA) i.e. molecules where the individual nucleobases were linked to an achiral backbone.

We now report herein a modification of PNA (eth B) in which the amide linkage to the nucleobase is replaced with tertiary amine. Our objective was to synthesise this PNA monomer and further to confirm effect on stability by its incorporation in a DNA sequence.

We have synthesise PNA monomer N-(t-Butoxycarbonylamino ethyl)-N (6-N-benzoyl, 9-ethyl) glycine in solution phase. This modification will eliminated the conformational constraint of the amide ad introduced the positive charge at the backbone point.

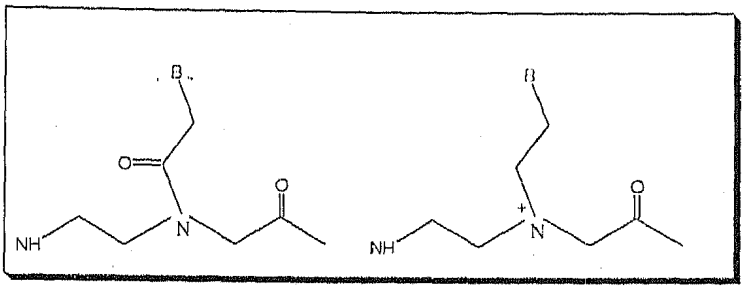


Fig. 2.8 Classical PNA monomer (on left), modified PNA monomer eth B (on right)

PNA forms highly stable complexes with DNA and RNA and the sequence discrimination is as good as or better in DNA hybrids. The improved hybridization between PNA/DNA has mainly attributed to the charge neutrality of PNA. Furthermore, the restricted flexibility imposed by the amide group is believed to be important for the favorable DNA mimicking properties PNA.

2.6 Results and discussion

A novel PNA monomer (N-(t-Butoxycarbonylamino ethyl)-N(6-N-benzoyl, 9-ethyl)glycine) have been synthesised in solution phase using Boc strategy. This modification is proposed to eliminate the conformational constraint of the amide and to make the backbone cationic.

The primary amino of adenine and cytosine were benzoylated and then these N-protected nucleobase were condensed with dichloromethane to yield 1-(ethylchloro) 4-N-benzoyl cytosine and 6-N-(Benzoyl)9-ethylchloro adenine as modified linker arm. On the other hand backbone N-(2-N-Boc) aminoethyl glycine was generated simply by condensing one primary amino functionality of ethylene diamine with chloroacetic acid and then protecting the other with Boc. Finally, the earlier prepared linker arm was attached to secondary amine group present in the backbone simply by condensation.

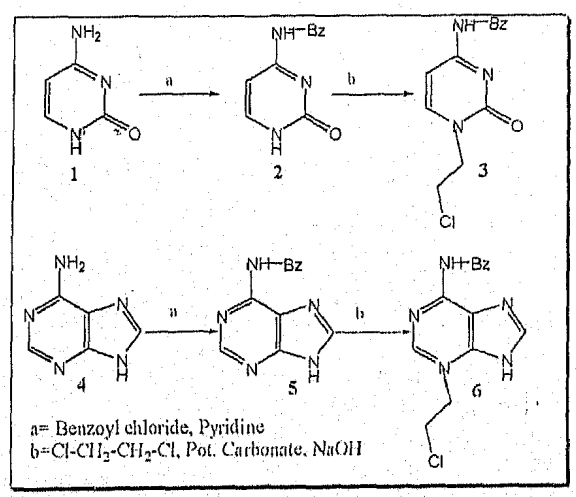
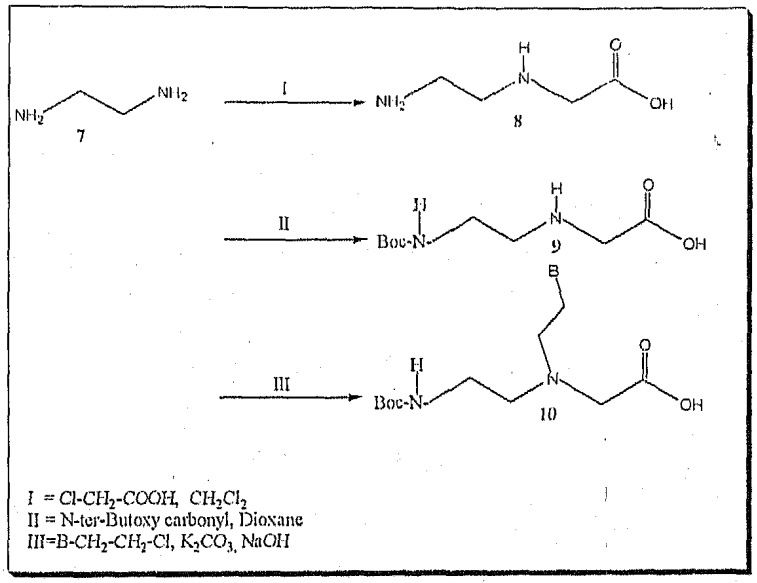


Fig. 2.8 Synthesis of linker arm

The modified PNA unit (eth B) will change both the conformational flexibility and the charge on PNA. The amide connecting the backbone and the linker to the nucleobase in modified PNA is a rigid unit due to the partial double bond character of C-N bond whereas its replacement in eth B. a tertiary amine is significantly more flexible. The tertiary amine is expected to be protonated at neutral pH and might therefore contribute to an electrostatic attraction between PNA/DNA.



Fg.2.9. Synthesis of backbone and linker arm attachment

The magnitude of attraction should be dependent on the distance between amine ad phosphate of the DNA backbone. Furthermore, it will be sensitive to changes in pH as well as changes in ionic strength. If the eth B unit contribute significantly to the electrostatic attraction between PNA and DNA this will be reflected in the relationship between Tm and pH, these results will be discussed in chapter four.

Experimental

4-N-(Benzoyl cytosine) (2)

To a suspension of cytosine (1.100 gm, 10 m mol) in about 100 ml of pyridine, 10 equivalent of benzoyl chloride (11.6 ml) was added dropwise. After completion of the reaction (after 2 hr.) reaction mixture was poured I water, and extracted with ethyl acetate (200 ml), ethyl acetate layer was concentrated in vacuo. finally crystallized in EtOH. Yield =70%, analytical calculation for $C_{11}H_8O_2$, C= 54.01%, H=3.21%, N=16.06% found, C=54.23%, H=3.10%, N=16.35%.

1-(ethylchloro) 4N-benzoyl cytosine (3)

To a suspension of 2 (554 mg, 2 m mol) in anhydrous DMF (5ml) was added anhydrous K_2CO_3 (276 MG, 2M MOL), after stirrig for 5 min., dichloroethane (.78 ml, 1m mol) was added dropwise. After stirring for calculated 23 hrs. The reaction mixture was evaporated in vacuo, and the residue was partitioned between ethyl acetate (35 ml) ad water (10 ml). The organic phase

washed with water ad brine , dried over $Na_2 SO_4$ and concentrated in vacuo. Purified by column chromatography (ethyl acetate/hexane, gradient elution), as white solid (50% yield). Analytical calculation , C=54.23, H=4.5, N=15.2,found, C=54.10, H=4.8, N=15.2

6-N-(Benzoyl adenine) (5)

To a suspension of adenine (1.351g,10 m mol) in about 100 ml of pyridine, 10 equivalent of benzoyl chloride (11.6 ml) was added dropwise. After completion of the reaction (after 2 hr.) reaction mixture was poured I water, and extracted with ethyl acetate (200 ml), ethyl acetate layer was concentrated in vacuo. finally crystallized in EtOH. Yield =70%, analytical calculation C= 55.44, H=4.62, N=23.10 found, C=55.20, H=4.71, N=23.2.

6-N-(Benzoyl) 9-ethyl chloro adenine (6)

To a suspension of 5 (538 mg, 2 m mol) in anhydrous DMF (5ml) was added anhydrous $K_2 CO_3$ (276 MG, 2M .MOL), after stirrig for 5 min., dichloroethane (.78 ml, 1m mol) was added dropwise. After stirring for calculated 23 hrs. the reaction mixture was evaporated in vacuo. And the residue was partitioned between ethyl acetate (35 ml) ad water (10 ml). The organic phase washed with water ad brine , dried over $Na_2 SO_4$ and concentrated in vacuo. Purified by colum chromatography (ethyl acetate/hexane, gradient elution), as white solid (50% yield). Analytical calculation , C=54.23, H=4.5, N=15.2,found, C=54.10, H=4.8, N=15.2.

2-amioethyl glycine (8)

to a vigorously stirred solution of ethylenediamine (3ml, 4.5mol) in dichloromethane (20 ml) at O^0C, was added chloroacitic acid (470 mg, 0.5 m mol) in dichloromethane (4 ml). The resulting reaction mixture was washed with water (3x50 ml) extracted with dichloromethane (50 ml). The combined organic layers were dried over Na_2CO_3 and filtered. Further dried in vacuo. Yield= 75 %, analytical calculation , C= 40.82, H= 8.47, N=23.72, found, C=40.82, H= 8.58, N=23.72., ^{1}HNMR 2.32 (S, 2H), 2.75(T, 2H), 2.62(T,2H.

N-(2-N-Boc) aminoethyl glycine (9)

To a suspension of 8 (202 mg, 2m mol) in a mix of 6 ml of dioxane and water, was added Boc anhydride (406 mg, 2 m mol). The mixture was allowed to warm at room temperature and the pH was maintained at 10.5 by the addition of aq. NaOH (2N). the solution was concentrated to paste which was triturated with DCM (50 ml). The suspension was filtered and organic phase was dried over $MgSO_4$, evaporated to give the product. Yield =90%, analytical calculation, C= 45.76. H= 9.32, N=17.79, found, C=45.50, H= 9.40, N=17.45.,

N-(t-Butoxycarbonylamino ethyl)-N(6-N-benzoyl, 9-ethyl)glycine (10)

To a suspension of 9 (438 mg, 2 m mol) in anhy. DMF(5ml) was added anhydrous $K_2 CO_3$ (276 mg, 2m mol), after stirring for 5 min., dichloroethane (1.56 ml, 2m mol) was added dropwise. After stirring for calculated 23 hrs. The reaction mixture was evaporated in vacuo. Ad the residue

was partitioned between ethyl acetate (35 ml) ad water (10 ml). The organic phase washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. Purified by column (ethyl acetate / hexane/ gradient elution), as white solid. Yield=65%, analttical calculation, C=64.58, H=5.70, N=13.87, Found, C=64.29, H=5.67, N=13.78, ^{1}HNMR, (DMSO) 10.68 (S,1H), 8.56 (d, 1H), 8.32 (s, 1H), 7.88 (s, 1H), 7.86(s,1H), 7.67(m,2H), 7.52-7.25(m, 9H), 5.34 (s,1H), 3.55 (T, 1H), 3.39 (m, 1H), 3.13 (q, 1H), 1.49 (s, 3H), 1.36 (s, 6H)

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CHAPTER-3

Labelling and synthesis of two heptamer sequences

5'-AAT GGA T-3'

&

*5'-A*TC CAT T-3'*

3. Introduction:

Modern molecular biology faces extremely complicated experimental problems. Proteins, polysaccharides, nucleic acids, and peptide nucleic acids, biological membranes and other ingredient of cell interact, form sophisticated structures and accomplish numerous catalytic, regulatory and other functions. In the investigation of biological systems, one uses a broad arsenal of physical and chemical methods. The necessity of such an approach is caused by the specificity of biological systems. The study of biomolecules usually tends to address following problems e.g.,

- What is precise structure at the atomic level of a macromolecule / or aggregate of molecules?
- What properties of macromolecules determine its structure and which forces participate in stablising this?
- If a macromolecule binds with other molecules, what is their structure? What is the number of bonding sites and what is the intensity of binding expressed in the form of physical constant?
- Where is particular macromolecule located within the cell or a small unit like virus?

It is therefore, rather than seeking complete information, a researcher usually aims to derive the main structural and dynamic properties important in the physical activity of the systems. Various methods based on colorimetery, electrophoresis, microscopic, immunological, hydrodynamic and spectroscopic properties are being used but of particular importance are biophysical labelling methods.

3.1. Biophysical labelling of Oligonucleotides / Peptide Nucleic Acids

Biophysical labelling involves selective modification of biological objects with various types of labels capable of providing information of their structure, molecular dynamics and mechanism of action. These days biophysical labelling methods are being used to solve a number of structural problems in various laboratories all over the world. This includes method of spin, triplet, photochromatic, electron scattering, Mossbauer and NMR, radioactive and fluorescent labelling[1]. The most used labels in investigation associated with oligonucleotide / Nucleic acids are the radioisotope on account of their high electron sensitivity. However, recently fluorescent labelling is being explored as a strong potential alternative to radioactive labelling is being explored as a strong potential alternative to radioactive labelling to for oligonucleotides/ nucleic acids.

3.1.1. Fluorescent labels

Fluorescent oligonucleotides/PNA's have recently attracted wide attention as probes for detection of nucleic acids hybridisation[2], DNA sequencing[3], and nucleic acid protein interaction[4]. Modified oligonucleotides are also useful as structural/diagnostics probes, therapeutic candidates and artificial enzymes[5-8]. Fluorescent detection means that the problem of stability, storage and disposal of radioactive compounds are avoided[9]. Furthermore, labelling of nucleic acids and peptide nucleic acids also made the techniques fluorescence *in situ* hybridisation (FISH), sequencing by hybridisation (SBH) as important tool for clinical diagnostics and gene mapping[10].

They are the prime requisite for exploiting the technology of chip-based DNA arrays prepared through combinatorial methodology[11] and also the fluorescence resonance energy transfer (FRET) [12] based detection has now become important tool for nucleic acid monitoring and identification with enhanced sensitivity of detection.

Fluorescent molecules represents another attractive class of reporter groups, which are being used for labelling of biomolecules particularly nucleic acids / peptide nucleic acids[13]. These are now largely replacing radioisotopes in biophysical labelling on account of following reasons.

- These molecules on excitation emit light in UV-VIS region and hence can be detected directly without requiring any procedural workup.
- Using fluorescence means that problems associated with radioisotope labelling viz. instability, health hazards and disposal problems can be avoided[14].
- The phenomenon of fluorescence itself is dependent on local environment (solvent / pH/ concentration etc.) and hence can be used as probe for molecular interactions[15], cellular functions and biochemical processes etc.[16].
- Since a number of fluorophores with discriminable spectra are available, it is possible to incorporate different fluorophores at different sites within the same molecule and detecting them simultaneously [17].

Besides, advent of multiple labelling[17], fluorescence resonance energy transfer (FRET)[18] and introduction of new hardware and detection system ensures that fluorescence detection can be performed with almost same level of sensitivity as radioisotope detection. Hood et al[19] were first to use automated nucleic acids sequencing systems using laser- based fluorescence detection and since then fluorescently labelled oligonucleotides/ peptide nucleic acids are being used for variety of biological application[20].

3.2. Application of Fluorescently labelled Oligonucleotides and / Peptide Nucleic Acids as Probes

Traditional probe technology uses small strands of deoxyribonucleic acids (DNA`s) or ribonucleic acids (RNA`s) called probes to diagnose disease, test for microbial contaminants or identify genetic elements. In these assays, the DNA or RNA probes are chemically synthesized and then mixed with the sample under investigation. Successful binding of the probes to nucleic acid molecules within the sample identifies the disease vector, microbe, or gene segment. As the name implies, PNA is related to, but distinct from, DNA. The difference between the chemical structure of PNA and DNA is simple, yet dramatic, in PNA a peptide-like backbone, and not a phosphate backbone, supports the nucleic acid bases. When PNA probes are put to the test, the result is simply a dramatic difference in the power of probe-based assays. The assays are faster, more accurate, and more sensitive. Target molecules that are impossible to identify using DNA or

RNA are readily accessible using PNA probes. In short, PNA has the power to transform traditional assays and enable the creation of entirely new assays[21].

3.2.1. Nucleic Acid Sequence Analysis and DNA Amplification

The structural analysis of DNA has an increasingly important role in modern Molecular biology. The detection, quantification and identification of nucleic acids have grown in importance due to the advent of Human Genome Mapping Project (HGMP) and nucleic acid based clinical diagnostics. The application of nucleic acid based clinical diagnostics require definitive information regarding nucleic acid sequences, a problem which is compounded by the vast dimension of genome. The human genome regularly contains about 10^5 genes encoded by 3-5 % of total 3x 10^9 base pairs of DNA.

The enzymatic method of sequencing developed by Sanger et al[22] and chemical method was elaborated by Mexam and Gilbert[23], these methods even though highly effective, suffer from the fact that these are labour intensitive, fairly expensive and involves the use of radioisotopes. Hood et al[13] was first to use fluorescence detection for nucleic acid sequencing and with some modification the method is now being used in sequence analysis of nucleic acid [24-28] and data so generated are being used for variety of diagnostic applications[29-35].

The fluorescence based sequence analysis is fairly accurate, cost effective and conventionally involve the use of fluorophore having emission in visible region. Since the emission in UV-VIS region may cause interference in sequencing samples and increased noise level due to autofluorescence of glass, solvents or impurities may reduce the potential sensitivity of the process, the use of Near-IR fluorophore is being contemplated in fluorescence based nucleic acid sequencing[36].

Similarly polymerase chain reaction (PCR) amplification of nucleic acids has become important and this is performed by using fluorescently labelled oligonucleotide primer to produce an amplified DNA product that can be detected and quantified absolutely[37-40].

MULTIPLEX PCR amplification of several nucleic acid sequences can be done using a pair of oligonucleotide primer labelled with different fluorophore. Each pair shall give rise to separate amplified products that can be identified due to their fluorescent labels[41].

3.2.2. PNA arrays

One important and critical aspect of array technology is the sensitivity and selectivity of the binding of the assayed DNA molecules. Also, DNA sequences form stable duplexes with arrayed DNA molecules only in the presence of ions which are needed to counteract the interstrand repulsion. Such conditions, however, also stablise secondary and tertiary structures with in a probe molecule. Sequence might therefore not be accessible and be prevented from hybridisation to the gridded DNA probe.

Peptide nucleic acids oligomer exhibits a much higher duplex stability with DNA than pure DNA-DNA hybrid do. More importantly, however, the PNA is an uncharged molecule. Because of the fact, DNA/RNA molecules can hybridise to PNA even in the absence of ions, thereby avoiding atleast in part the sterically caused prevention of binding. In addition as an artificial molecule, PNA is not degraded by nucleases or proteases.

Last the structural difference between PNA probe and DNA- target allows for detection method that can be much more simple and sensitive than the current techniques[21].

3.2.3. Fluorescence *in situ* Hybridisation

In situ hybridisation is a technique, which allows the detection and location of given nucleic acid directly in cell smears, and tissue section. The slides are incubated in hybridisation buffer at elevated temperature so as to denature the cellular DNA which is followed by hybridisation with a probe, when the probe is fluorescently labelled the technique is called fluorescent *in situ* hybridisation (FISH) [42] . It can be used to locate genes on the chromosome or even detect chromosomal abnormalities.

Recent works suggest the possible use of FISH in identification and detection of micro-organism[43], human chromosome centromere identificationn[44], chromosome examination in human blastosoma[45], human papiloma virus[46]. Now, probes are available which fluoresce only after hybridisation. These are called Molecular Beacons[47]. Since multiple and simultaneous fluorescence detection is possible, there is a potential to visualise several fluorescent signals relating to different genetic sequences within the sample[48].

A PNA probe based assay offers more accurate, less time consuming method for the detection and length measurement of telomeres. In this FISH procedure[49], individual metaphase spread and interphase nuclei are the basis for analysis. An octadecameric PNA probe $(CCCTAA)_3$ is used for the hybridisation. The probe does not recognise sub- telomeric sequences; therefore, telomeic length is directly correlated with the fluorescent intensity of the spot and quantitation is extremely accurate[50].

3.2.4. The Lightup Probes

Lightup probes have been constructed for the specific nucleic acid detection in homogenous solution[51]. The light up probe is a PNA linked to a asymmetric cyanine reporter dye. When added to a sample, the light up probe binds to complementary nucleic acid becoming intensively luminescent. Designing the lightup probe with a melting temperature between the annealing and elongation temperature of the PCR reaction, the lightup probes can be present during PCR reporting the amount of form in real time. Owing to very high affinity of PNA for nucleic acids, the lightup probe out-competes efficiently reannealing of the ds-DNA product. Using lightup probes in real time PCR we can determine the number of DNA molecules in biological samples

with a precision +/- 20% with a sensitivity that only is limited by sample preparation and the contamination problem.

3.2.5. Chemiluminiscent in situ Hybridisation

Chemiluminiscent in situ Hybridisation method is performed on a membrane. The sample is filtered onto a membrane to isolate and separate individual microorganism. The membrane is placed on a culture medium typically for one-third of the time required to obtain visible colonies prior to testing. Microcolonies are detected on the membrane by *in situ* hybridization using peroxidase-labeled PNA probes. Excess probe is removed by washing, and the hybridized probes are visualized by chemiluminescent reaction followed by film exposure. Each colony can be observed. In conclusion, the PNA CISH assay affords a rapid method for unequivocal and simultaneous detection identification and enumeration of bacteria[52].

3.2.6. DNA Fingerprinting

DNA fingerprinting[53] is a fast growing technique which is being used to detect and analyse gene mutation, quantitative mutation analysis, short tendam repeat polymorphism (STRPs) etc. which can be used for gene mapping, genetic diagnostics, forensic analysis and paternity testing etc. A method for the analysis of DNA fingerprinting using fluorescently labelled Oligonucleotide probe has been debveloped[54]. This has been used to detect overlapping frequency of DNA fragments in cosmid clones of mouse from MHC class-I gene culture[54]. This technique involving fluorescently labelled probes for DNA fingerprinting is called Fluorescent Fingerprinting (FF) technique which has been shown to detect gene mutation[55] and quantitative mutation analysis of mutation of mitochondrial DNA which can be used to detect heteroplasmic point mutation associated with Alzheimer's disease[56] besides, detection of STRPs using fluorescent probes has also been established[57].

3.3 Fluorescent Labelling of Oligonucleotides

Covalent attachment of fluorophore to oligonucleotides can be carried by a variety of enzymatic[58-61] and /or chemical[62-65] methods. Moreover, this may be carried out at different sites like, purine/ pyrimidine bases, phosphate backbone and 2'-OH and 3'-OH/ 5'-OH terminal of oligonucleotides. Covalent attachment of fluorophores at bases generally involves modification of bases to generate active sites such as aliphatic amine at N-4 in cytidine by a bisulphite catalysed transmination reaction[58,66] or C-5 of uridine by palladium catalysed olifination reaction[67-68]. Similarly, fluorescent labelling can also be carried out at phosphate backbone, for instance by condensing terminal phosphate by a bifunctional amine to give (aminoethyl) phosphoramidite which can then be reacted with fluorophore bearing amino functionality[69]. Fluorescent labelling at internucleotide phosphate by solid phase synthesis has been reported[70,71]. Of all the labelling strategies however the most frequently used one are which involves 5'-OH of nucleotides and oligonucleotides.

Various methods being used to carry out fluorescent labelling at various position of oligonucleotides has recently been reviewed[72] and hence in following section only 5'- labelling strategy involved in present work shall be discussed in detail.

The 5'-OH labelling strategies can be of two types viz. Automated and manual.

3.3.1. 5'-OH Automated Labelling

Automated labels[73] incorporation is the most direct route to oligonucleotide possessing fluorescent groups at 5'-position, and is probably the most widely used of all labelling strategies. Fluorescent phosphoramidites can be coupled to the 5'-terminus of synthetic oligonucleotide at the last stage of synthetic sequences. By increasing the reaction time specified by the automated synthesiser programme will reduce possible problems of lower reactivity due to increased steric bulk of fluorescent phosphoramidite. Commercially available phosphoramidites of fluorochromes based on fluoresceine, tetrachlorofluorescin, hexachlorofluoresceine, and rhodamine are shown in FIG. 1

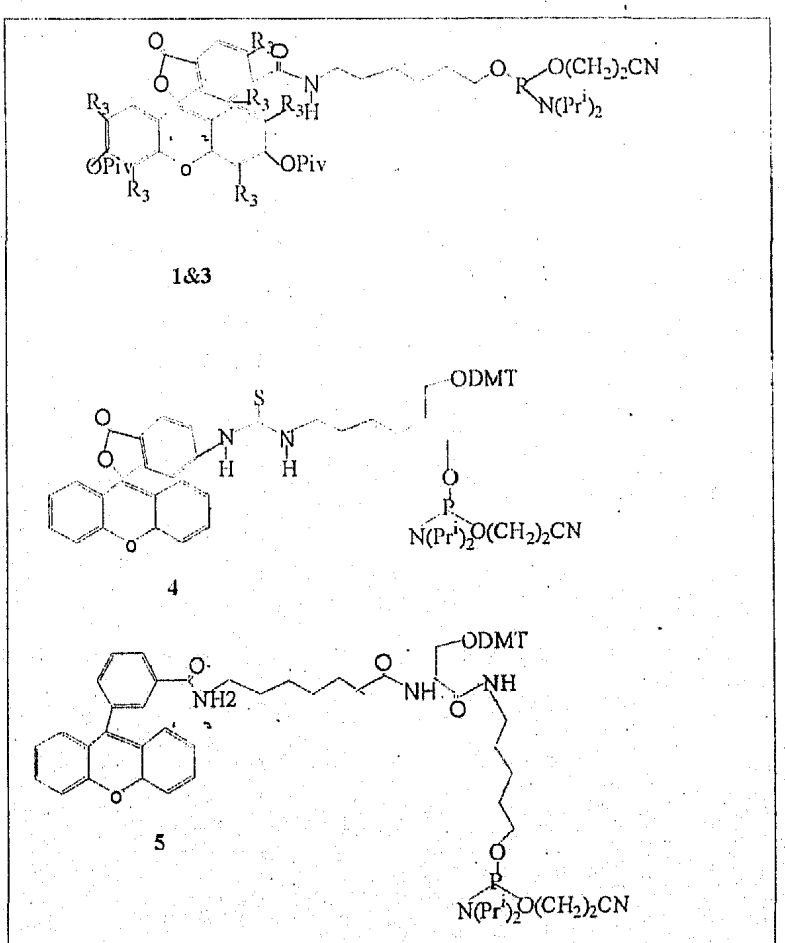


FIG.1 Commercilly avaialable fluorescent amidites;1 & 4 fluorescein; tetrchlorofluorescein, 3 hexachlorofluorescein;5, rhodamin

It can be observed from Fig.3.1 possess extra arm terminating in a DMT- protected hydroxy group, which can be removed to anchor more than one fluorochrome. If however, more specialised

prerequisite is that label should have primary/ secondary hydroxyl function which can be converted to the cynoethyl phosphoramidite, using a easily available commercial phosphitylating agents such as $ClP(OCH_2CH_2CN)N(Pri)_2$ which is readily available commercially.

3.3.2. Manual 5'-OH Labelling

The greatest advantage of manual labelling is that a far great variety of labels can be conjugated to oligonucleotides. A large number of specific phosphoramidites for introduction of flurochromes are commercially available[74-77]. Various kinds of 5'-end labelling strategies have been discussed below.

3.3.3) Via 5'-Amino Functionality

The fluorescent labelling of an oligonucleotide at the 5'-terminus by the route usually involves two steps, and the most commonly proceeds as follows. An N-protected aminoalkyl phosphoramidite

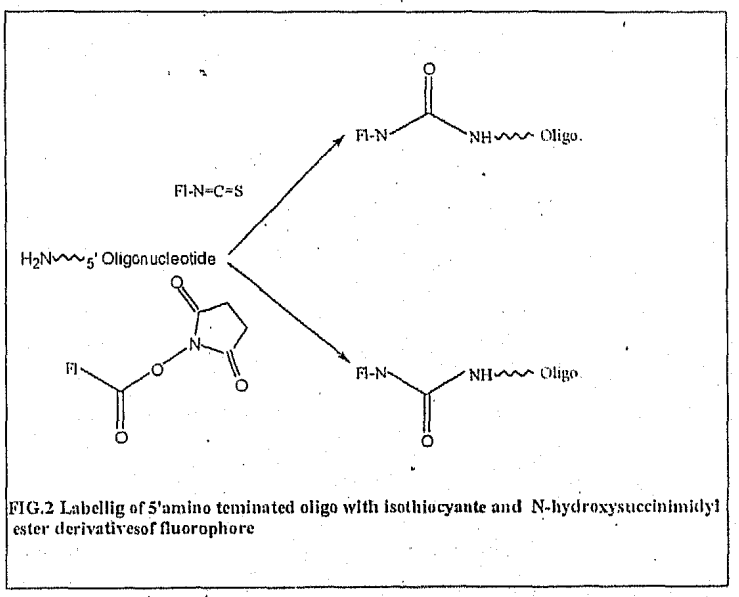


FIG.2 Labellig of 5'amino teminated oligo with isothiocyante and N-hydroxysuccinimidyl ester derivativesof fluorophore

derivative is coupled to the 5'-terminus of oligonucleotides at the final stage of automated DNA synthesis. Following cleavage and deprotection, an appropriately substituted fluorescent dye can then be manually coupled to 5'- amino group. Typical derivatives are isothiocyanate and N-hydroxysuccinimidyl esters (NHS) with later being more favored as they are less prone to competing hydrolysis in aqueous, basic reaction medium, thus giving higher yield of labelled oligonucleotides.(Fig.3.2)

Also the thiocyanate linkage formed by the reaction with ITC is prone to degradation during long term storage[78].

Many phosphoramidite containing protected amino group have been developed in order to generate aliphatic amino group at the 5'-terminus of oligonucleotides after cleavage and deprotection.(Fig.3.3). Treatment with ammonia (post synthesis) and mild acid treatment can remove the trifluroacetyl group/ monomethoxytrityl group in above example respectively. If the ITC or NHS ester derivatives are

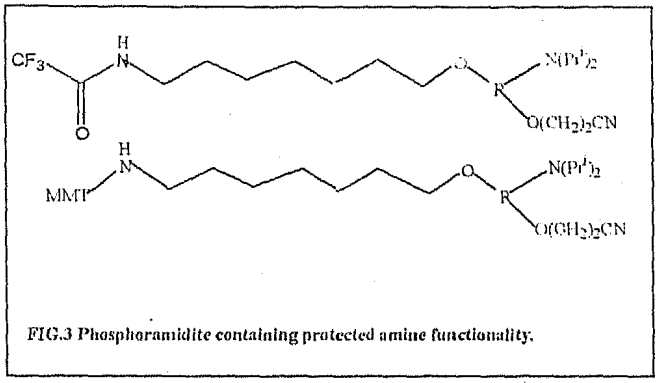


FIG.3 Phosphoramidite containing protected amine functionality.

not available, many other alternatives can be explored[79] similarly if phosphoramidites with protected amino group are not to be used, then alkylamino functionality can be generated in many other ways like reaction of diamines with 5`- phospharimidaxolides[63,80] (acid prone linkage) or reaction of 2-(biotinylamide) ethanol with 5`-phosphorylated oligomers[62] or reaction of alkyldiamines in presence of CDI[81]. The choice infact varies as per requirement.

3.3.4. Via a 5'-phosphate or 5'-hydroxy functionality:

5`-Phophatorylation, followed by amination has been a popular method for introducing functionality, which can be modified with a fluorochrome[82]. An obvious extension of this approach, which avoid the need for what is essentially, a three step labelling strategy is to use a fluorochrome that has already suitable amino funtionalittyn attached (Fig.3.4).

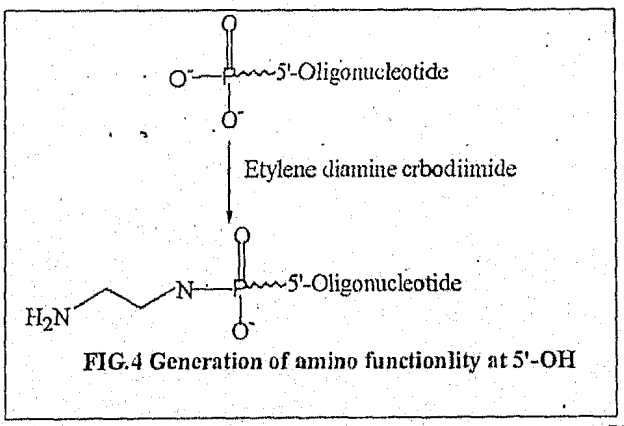


FIG.4 Generation of amino functionlity at 5'-OH

This can be prepared in laboratory or are commercially available [78](ethylamine /cadiverine derivatives).

3.3.5. Via a 5'-Thiol Functionality:

The thiol (-SH) functional group can be used to conjugate oligonucleotides with reporter group under the similar reaction condition to those used with 5'-amino terminated oligonucleotides.

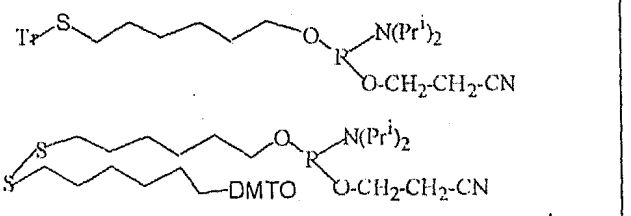


FIG.5, Some Commercially available phosphormidites

Instead of isothiocynate (ITC) or N-hydroxysuccinimidyl (NHS) derivatives. maleimoide or iodoacetamide derivative of fluorochrome are used. Typical commercially available phoramidites[83] in this category are shown in (Fig. 3.5) .

3.4. Fluorescent Labelling of PNA

Labelled PNA can be prepared either by using Fluorophore derivatised monomer in the coupling protocol or by modification of N- terminal amino group[84-87]. The former method requires the preparation of respective unnatural monomers and the later results in the inevitable loss of the reactive terminal amino group of PNA, which prevents further modification of PNA through the amino group.

The strategy for C-terminal labelling is based on thiol chemistry employing cysteine as the key C-terminal linker to provide a SH- functionality[88]. Efficient thiol deprotection and its subsequent coupling with a fluorophore on solid phase was sought for ease of purification of the labelled PNA derivative. The cysteine-thiol group was protected as disulfide with S- t- butylmercapto group which provide the required orthogonality to the base labile Fmoc group and all the acid labile protecting groups on the nucleobase of PNA monomer. Fully protected PNA was prepared on Tentagel resin, pre-loaded with S- t- butylmercapto- L- cysteine via a Rink amide linker, using literature based on Fmoc chemistry[89].

To further expand the utility of chemistry, a dual labelled PNA was prepared by solid phase chemistry with Tetramethylrhodamine at the N-terminus (via NH_2) and fluorescein at the C-terminus (via a cysteine) . Following the Fmoc deprotection of PNA. The terminal NH2 group was reacted with TMR-succinimidyl ester to give singly labelled resin bounded PNA. The S- t-butylmercapto group was deprotected by dithiothreitol, and the thiol was reacted with 5-iodo-acetamidofluorescein in DMF. Isomeric products resulting from TMR were composed of 22 and 10% respectively, of crude material following cleavage[90].

This demonstrates that it is possible to use the $-NH_2$ and –SH functionalities for the dual derivatisation of PNAs. Labelled nucleic acids, e.g. molecule beacons[91] have widely been used as diagnostic tools for biomedical studies.

3.5. Present work

A number of synthetically prepared oligonucleotides and peptide oligomers are recently under going human clinical trials for the treatment of variety of cancerous, viral disorders and host of inflammatory disorders. With growing success of clinical programs and potential market demands for oligonucleotide based drugs, demand of synthetic oligomers/ PNA oligomers and consequently fluorescently labelled oligomers is increasing. Of all the processes developed so far, phosphoramidite building blocks are being preferred on account of high coupling efficiency and rapid turn over time[92].

In the present work we have synthesised and labelled two heptamer DNA sequences,

5`-*flu*AAT GGA T-3` (*flu*=fluorescein) and its complementary sequence

5`-*rh*A* TCC ATT-3` (*rh*=rhodamine, A* denotes PNA monomer with adenine as base)

The fluorescein unit is attached at the 5'-of the sequence (5'-AAT GGA T-3) by using 5-aminopentanol as linker. The fluorescein was first attached with 5-aminopentanol unit and the free hydroxyl terminus was phosphitylated. In the last coupling cycle of DNA sequence synthesis amidite was coupled to give 5'-*flu*-AAT GGA T-3`.

Step	Reagents and Solvents	Function	Time (min)
1	Anhydrous acetonitrile	Wash	2
2	3% Trichloroacetic acid in dichloromethane	Detritylation	0.5-1.5
3	Anhydrous acetonitrile	Wash	2
4	Nucleotide, ^1H-tetrazole, anhy. Acetonitrile	Coupling Step	15
5	Repeat step 1-4 until the sequence is complete In the last cycle rhodamine phosphoramidite was coupled		20
6	0.1M iodine/pyridine/water (90/5/5)	Oxidation of Phosphoramidite	10

TABLE 3.1 Phosphoramidite synthesis cycle

5`-*rh*-A*TC CAT T-3` is a labelled and modified sequence, the sequence was synthesised using phosphoramidite approach (table 4.1). The A* unit represented the modified PNA monomer having adenine as nucleobase. To this 5'-A*TC CAT T-3' fluorescent group attached is rhodamine. The rhodamine was first attached with a two carbon linker using ethanolamine. The free -OH terminus was further used to generate its phosphoramidite by using pyridinium trifluoroacetate and N,N,N',N'- bis tetraisopropyl phosphoramidite. The usual procedure for preparing amidite building blocks involve phosphitylation of free hydroxy group with 2-cyanoethyl-N, N-diisopropylchlorophosphoramidite in presence of activator 1H tetrazole. Since, 2-cyanoethyl-N, N, N', N'-diisopropylphosphoramidite (bis reagent) is fairly stable and less expensive, therefore, it has been used in the present work.

3.6. Result and Discussion

In the present work we have synthesised and labelled two heptamer DNA sequences,

5`-*flu*AAT GGA T-3` (*flu*=fluorescein) and its complementary sequence, 5`-*rh*A* TCC ATT-3` (*rh*=rhodamine, A* denotes PNA monomer with adenine as base)

Covalent labelling of fluorescein 5`-AAT GGA T-3`with DNA sequence was carried out by preparing the amidites of 1-(fluorescenylamido)-pentan-5-O-(cyanoethylphosphoramidite)

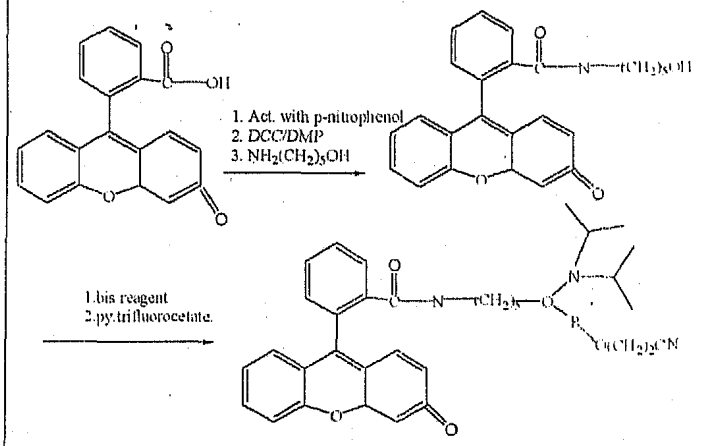
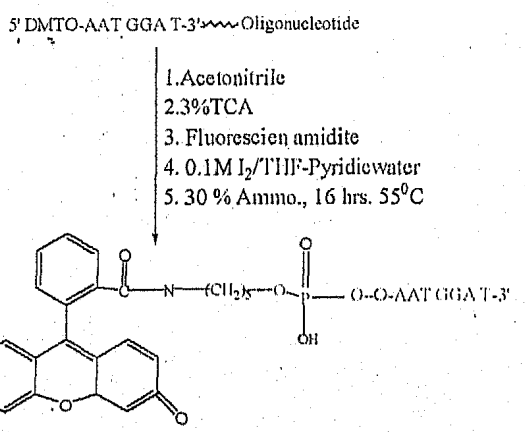


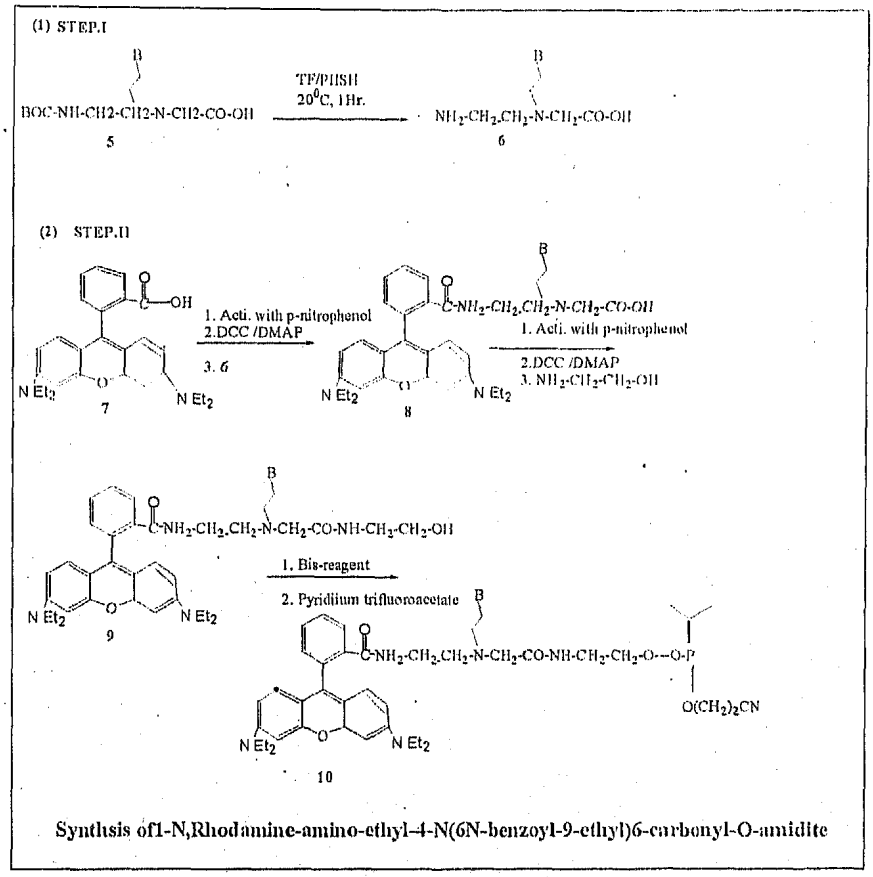
FIG.6Synthesis of fluorescein amidite



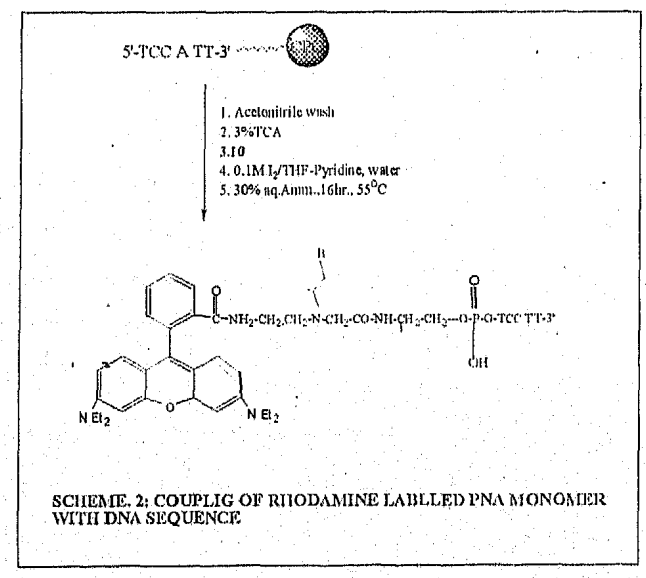
SCHEME 1

(**scheme 1**) The fluorescein unit is attached at the 5`-of the sequence (5`-AAT GGA T-3) by using 5-aminopentanol as linker. The fluorescein was first attached with 5-aminopentanol unit and the free hydroxyl terminus was phosphitylated. In the last coupling cycle of DNA sequence synthesis amidite was coupled to give 5'-*flu*-AAT GGA T-3'.

Amidite of rhodamine 1-N- rhodamine-4-N-(ethyl-adenine)-6-carbonyl-ethane-O-amidite (Scheme 2). The rhodamine was first attached with a two carbon linker using ethanolamine. The free -OH



terminus was further used to generate its phosphoramidite by using pyridinium trifluoroacetate and N,N,N',N'- bis tetraisopropyl phosphoramidite. After phosphitylation this amidite was condensed



with PNA monomer, which is further coupled with DNA sequence 5'-TCC ATT-3'.

3.7. Experimental

Materials And Methods

All Solvents used were of Qualigens analytical grade, which were further purified and dried prior to use. 2-Cyanoethyl-N, N, N', N'-diisopropylphosphoramidite (bis reagent) and pyridinium trifluoroacetate were obtained from ISIS pharmaceuticals. Synthetic oligonucleotide was obtained from Hybridon Inc. USA. 1H-tetrazole and trichloroacetic acid were purchased from Sigma Chemicals company, USA. UV was recorded on Hitachi 220S UV- Visible Spectrophotometer. Purification of labelled oligo's was carried out by HPLC using C_{18} column employing UV-VIS detector.

3.7.1. Synthesis of 1-(fluorescenylamido)-pentan-5-O-(cyanoethylphosphoramidite) (scheme 1):

(I) 5-(fluorescenylamido)-pentanol-1

Fluorescein (2 mM, 664.62 mg) was dissolved in dioxane (15ml) to its stirred solution p-nitrophenol (2.4mM, 333.9 mg) was added followed by 2 eq. Of DCC and 0.05 eq. of DMAP. After 2 hr. 5-aminopentanol (2 mM, 217.42 mg) was added in equimolar ratio with further addition of 2eq. of DCC. The reaction mixture was stirred for another 5 hr. at r.t. Completion of reaction was checked on tlc with a new spot (Rf, 0.04, fluorescein has Rf of 0.3 in pure DCM). The reaction mixture was evaporated in vaccuo and then loaded on a short silica column (80-120 mesh; 6x2 cm.). the desired product was eluted with 40: 60 hexane: DCM, yield = 58%.

(II) 5-(fluorescenylamido)-pentanel-5-O-phosphoramidite:

(1.5 mmol, 626.3mg) of fluorescein amide-1-pentanol was suspended in DCM (15ml). To it bis reagent (3mmol, 1ml) was added followed by pyridinium trifluoroacetate (3mmol, 600mg) at ambient temperature under argon. After 4 hr. the reaction mixture was checked on tlc. (DCM irrigating solvent). The product spot was found at (Rf= 0.52). The reaction mixture was concentrated in vaccuo and purified on silica by column chromatography.

3.7.2 Covalent Attachment of amidite of fluorescein to 5'-AAT GGA T-3':

The polymer attached sequences were taken in a functionalization vessel having frit and a supply for uninterupted argon. The CPG was washed with dry acetonitrile and the 5'-O-DMTr group was deprotected by 3% Trichloroacetic acid for 3 min. The column was washed twice with dry actonitrile. The fluorescein amidite was dissolved in dry acetonitrile was mixed with tetrazole and added to the vessel. A coupling time of 20 min. was allowed, followed by washing with acetonitrile. Oxidation of the sequence was done using I_2 in THF/ Pyridine/ water (90: 5:5) for 15 min. The column was again washed with acetonitrile and the resin was transferred in 30% aq. Ammonia and incubated at 55^{0}C for 8 hrs. The ammonia solution was filtered again through frit to remove the polymer and evaporated in vaccuo. The desired sequence was precipitated and desalted using isopropanol. The pellet obtained after centrifugation were stored in deep freeze at –30^{0}C.

3.7.3. Synthesis of 1-N, rhodamine-amino-ethyl-4-N(6N-benzoyl-9-ethyl) 6-carbonyl-ethane-O-amidite (scheme2):

(I) Synthesis of 1-N, rhodamine,-4-N(benzoyl-9-ethyl) glycine:

Boc group of PNA monomer (N-(t-butoxycarbonyl)amino ethyl-N(6-N-benzoyl, 9-ethyl) glycine was deblocked with TFA and thiophenol at 20^{0}C for 1 hr. Rhodamine (2 mmol, 800 mg) was dissolved in dioxane (15ml) and to its stirred solution p-nitrophenol (2.4mmol, 333.9 mg) was added followed by DCC (2 eq.) and DMAP (0.05 eq.). After 2 h N-2-amino ethyl-N-(benzoyl-ethyl adenine) glycine was added in equimolar ratio with further addition DCC (2eq.). The reaction mixture was stirred for another 5 hr. at r.t. Completion of reaction was monitored on tlc with a new spot (Rf.= 0.6, rhodamine has Rf of 0.4 in pure DCM).

The reaction mixture was evaporated in vaccuo and then loaded on a short silica column (80-120 mesh; 6x2 cm.). The desired product was eluted with 40: 60 hexane: DCM, yield = 65%.

(II) Synthesis of 1-N-rhodamine-4-N-(benzoyl-9-ethyl)-6-carbonyl-ethanol amine:

1-N, rhodamine,-4-N,(benzoyl-9ethyl) glycine (2 mM) was dissolved in dioxane (15mL). To its stirred solution p-nitrophenol (2.4mM, 333.9 mg) was added followed by DCC (2 eq.) and DMAP (0.05 eq.). After 2h ethanol amine was added in equimolar ratio with further addition of 2 eq. of DCC. The reaction mixture was stirred for another 5 hr. at r.t. completion of reaction was monitored on tlc with a new spot [Rf.= 0.8, 1-N, rhodamine,-4-N,(benzoyl-9ethyl) glycine] has Rf of 0.6 in pure DCM). The reaction mixture was evaporated in vaccuo and then loaded on a short silica column (80-120 mesh; 6x2 cm.). The desired product was eluted with 40: 60 hexane: DCM, yield = 60%.

(III) Synthesis of 1-N-rhodamine-aminoethyl-4-N-(6N-benzoyl-9-ethyl)-6-carbonyl-ethane-O-amidite:

(1.5mM) of synthesis of 1-N- rhodamine-4-N-ethyl-adenine-6-carbonyl ethanol amine was suspended in DCM (15mL). To it bis reagent (3mM, 1mL) was added followed by pyridinium trifluoroacetate (3mM, 600mg) at ambient temperature under argon. After 4 h the reaction mixture was checked on tlc. (DCM irrigating solvent). The product spot was found at (Rf= 0.6). The reaction mixture was concentrated in vaccuo and purified on silica by column chromatography.

3.7.4. Covalent attachment of amidite of Rhodamine to 5'-A*TC CAT T-3':

The CPG loaded thymidine was taken in a functionalisation vessel having frit and a supply for uninterupted argon and the sequence was synthesised with standard phosphoramidite approach[94]. The CPG was washed with dry acetonitrile and the 5'-O-DMTr –group was deprotected by 3% trichloroacetic acid for 3 min. The column was washed twice with dry acteonitrile. The rhodamine amidite was dissolved in dry acetonitrile and was mixed with equimolar amount of 1H-tetrazole and added to the vessel. A coupling time of 20 min. was allowed, followed by washing with acetonitrile. The sequence is oxidised using I_2 in THF/ Pyridine/ water (90: 5:5) for 15 min. The column was again washed with acetonitrile and the resin was transferred in 30% aq. Ammonia and

incubated at 55^{0}C for 8 hrs. The ammonia solution was filtered again through frit and evaporated in vaccuo. The desired sequence was precipitated and desalted using isopropanol. The pellet obtained after centrifugation were stored in deep freeze at –30^{0}C.

Note: A*_denotes PNA monomer containing adenine as base.

Calculation of loading of nucleosides on LCAA-CPG

Loading of Nucleosides = A498 X Vol. / 76 x 1000 / wt. (μM) of starting resin

Absorbance (495 nm) of trityl solution = 2.310 OD

Vol. Of trityl solution = 5 ml

Total absorbance = 2.310 x 5 = 11.550

Therfore, loading of nucleosides = 11.550/ 76 x 1000/ 4

= 11550/ 304

= 38.4 μM / g CPG

Calculation of overall yield (Y_0)

For DNA sequence (5'-A*TC CAT T-3')

$$= \frac{\text{Total OD x 100}}{[(15.4 \times nA) + (8.8 \times nT)] \times \mu M / g\ CPG}$$

$$= \frac{1125.772 \times 100}{[(15.4 \times 2) + (8.8 \times 3)] \times 2}$$

$$= \frac{112577.2}{[(15.4 \times 2) + (8.8 \times 3)] \times 2}$$

$$= \frac{112577.2}{132}$$

= 93%

For DNA sequence (5'-AAT GGA T-3')

$$= \frac{\text{Total OD x 100}}{[(15.4 \times nA) + (8.8 \times nT)] \times \mu M / g\ CPG}$$

$$= \frac{1125.772 \times 100}{[(15.4 \times 2) + (8.8 \times 3)] \times 2}$$

$$= \frac{112577.2}{[(15.4 \times 2) + (8.8 \times 3)] \times 2}$$

$$= \frac{112577.2}{132}$$

= 90%

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CHAPTER-4

Comparative hybridisation studies of two labelled heptamer sequences

4.1 Introduction:

Hybridisation is a term used to describe the specific complementary association due to hydrogen bonding, under experimental conditions, of single stranded nucleic acids. It should more properly be referred to as "annealing", as this is the physical process responsible for the association. The two complementary sequences form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid" helical[1] molecule.

In normal double stranded duplexes Watson-Crick base pairing is found, their geometry is such that any sequence of base pair can fit into nucleic acid helix without distortion (Fig.4.1), although, purine and pyrimidine have different geometry. In Watson-Crick base pairing C-6 position and N-1 position of adenosine is associated with N-3 and C-4 functions of thymine .

Each nucleic acid helix has a major and a minor groove resulting from their secondary structures. The formation of unusual secondary structure, such as cruciform, triple stranded, junctions and so on in DNA (sometimes induced by negative supercoiling) are one important cause of unpaired bases.

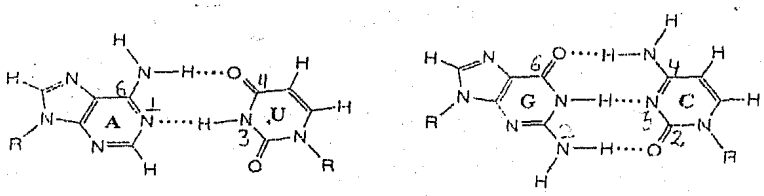

FIG.4.1 Watson and Crick base pairing

4.1.1Triple strand formation

Triplex formation is based on recognition of Watson-Crick hydrogen bonded AT and GC

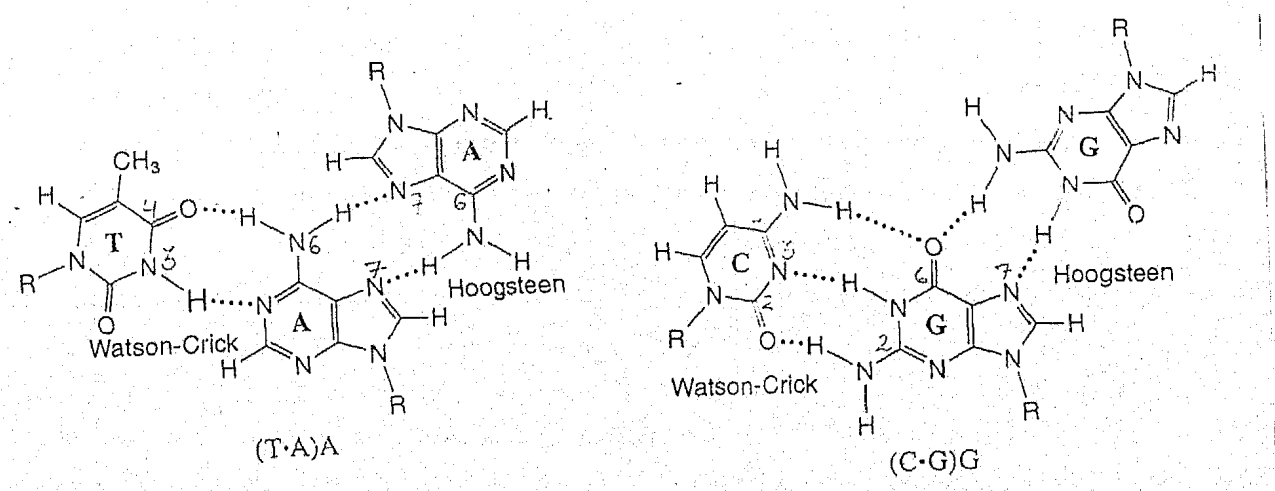

FIG. 4.2 Base pairng pattern in triplex formation

base pairs in duplex by a third strand T and C^+ respectively via Hoogstein hydrogen bonds (Fig.4.2). Since in Watson-Crick base pairing C-6 position and N-1 position of adenosine associate with N-3 and C-4 of thymine, therefore still N-7 of adenosine is available for hydrogen bonding and this takes part in Hoogstein pairing.

4.1.2 The complementary association of two strands of polynucleotides

The complementary association of two strands of polynucleotides is the basis for replication of all organisms; the complexity inherent in the sequence of the molecules renders the association extremely specific for any molecule longer than sixteen nucleotides. This is easily understood if one considers the combinatorial possibilities of given lengths of "probe" sequence: there is a ¼ chance (4-1) of finding an A, G, C or T (U for RNA) in any given DNA sequence; there is a 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a 1/256 chance of finding a given 4-base sequence. Thus, a sixteen base sequence will statistically be present only once in every

416 bases (=4 294 967 296, or 4 billion):

this is about the size of the human genome, and 1000x greater than the genome size of *E. coli*.

Thus, the association of two nucleic acid molecules presumed to be at least a few hundred bases long is an extremely sequence specific process, far more so than the widely used specificity of monoclonal antibodies in binding to specific antigenic determinants. The correct annealing of two sequences to each other does, however, depend on the physical and chemical solution conditions under which the reaction takes place.

4.2 PNA / DNA interactions

PNA is a remarkable mimic of nucleic acid in which the phosphodiester backbone is replaced by a pseudo –peptide chain. PNA bind to complementary DNA or RNA by Watson-Crick base pairing to form duplexes of high stability (Fig.4.3). In contrast to DNA they can however, bind in both parallel and antiparallel orientation. In PNA its C-terminus corresponds to 5' end of the normal oligonucleotides and the N-terminus to 3'-end of normal oligonucleotides. Antiparallel orientation is favored and (antiparallel PNA/DNA) are considerably more stable than the corresponding DNA/ DNA complexes. DNA has strong binding affinity to PNA, even higher than its complementary strand and hence PNA's are ideal to target DNA within the nucleus and homopyrimidines PNA forms triplexes. Homopyrimidine PNA and PNA oligomers with high pyrimidine / purine ratio binds to complementary DNA normally by formation of unusually stable $(PNA)_2$/DNA triplex.

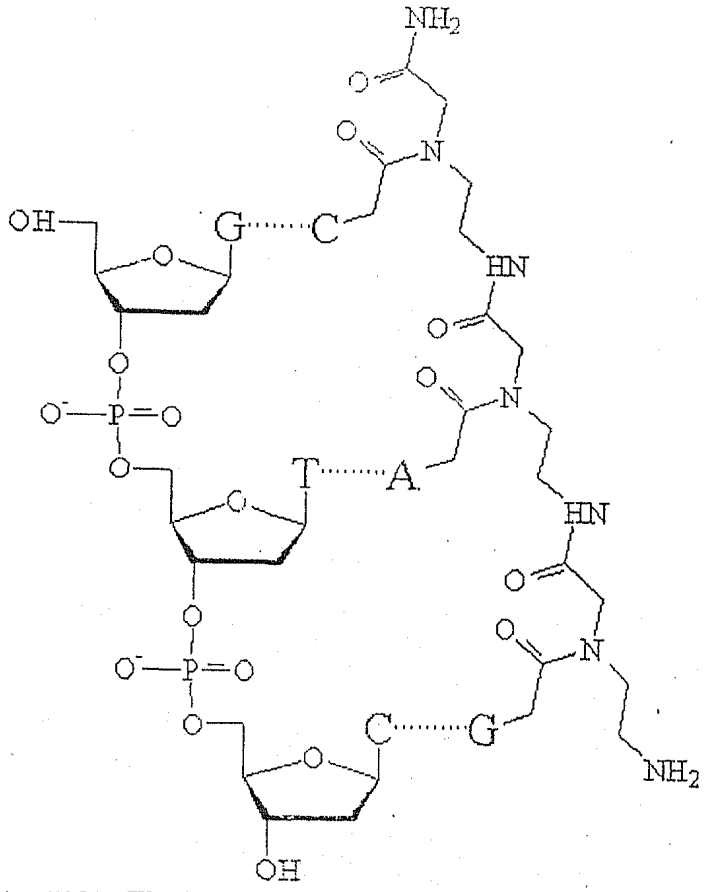

FIG. 4.3 DNA – PNA: The interaction is according to normal Watson Crick pairing .

4.2.1 Strand invasion

It has been found that PNA invades (p-loop formation) DNA duplex in sequence specific manner, displacing the existing DNA complementary strand. Two simple mechanisms of strand invasion

FIG.4.4 P-loop. P-loop structures formed by strand invasion of homopyrimidine mono-PNAs (A) or bis-PNAs (B) into dsDNA.

As shown in Fig. 4.4, Homopyrimidine PNAs (A) contain only C and T as nucleobases, and in Bis-PNAs (B) are two homopyrimidine PNAs connected by a flexible linker molecule. Two mechanism of strand invasion are possible. In one mechanism, the first stage consists of fluctuational opening of the DNA double helix and in a transient formation of a PNA/DNA Watson-Crick duplex (the Watson-Crick-first mechanism). In the other possible mechanism, the first stage consists of formation of an unstable DNA/DNA/PNA

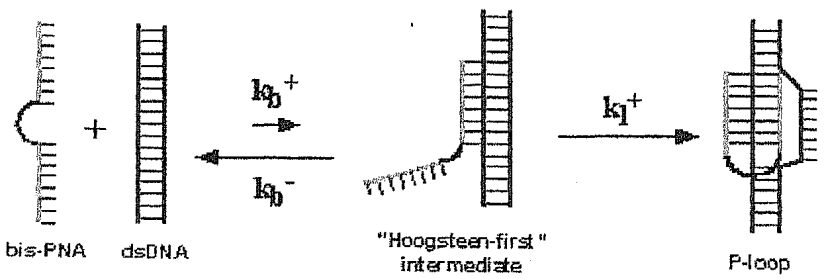

FIG. 4.5. Proposed Hoogsteen-first mechanism at strand invasion of homopyrimidine bis-PNAs into ds (double stranded) DNA

triplex via Hoogsteen pairing (the Hoogsteen-first mechanism). Since the final result is the same for both mechanisms unstable intermediates are very difficult to detect, the particular route of P-loop formation remained elusive.

4.2.2 Hybridisation of Peptide nucleic acid probes to Structured DNA/RNA targets

The use of synthetic DNA analogues to recognise and bind (i.e. hybridise) to complementary

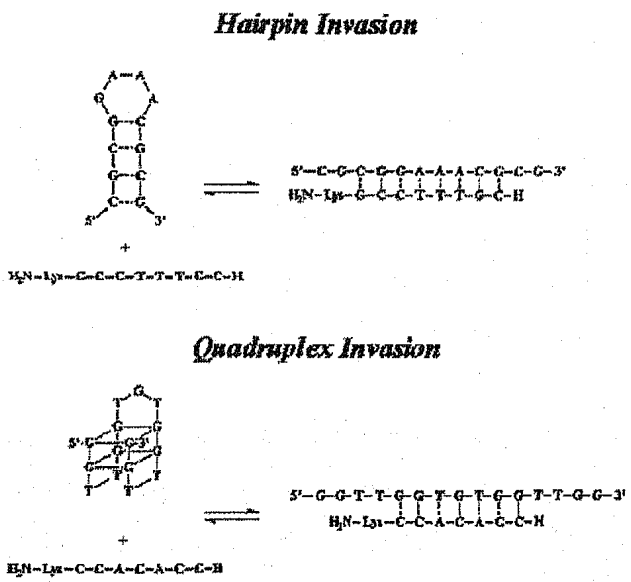

FIG.4:6 Hairpin and Quadruplex invasion

sequences in DNA /RNA[33] is the basis for nucleic acid diagnostics, genomics analysis and antisense/ antigene therapy[34]. While it is straight forward to use Watson-Crick rules to design a hybridisation agent for a particular target sequence, achieving successful hybridisation is much more complicated. One of the biggest obstacle is folding of target nucleic acid. Thus the three dimensional shape of the target, defined by the secondary and tertiary structure, imposes kinetic and thermodynamic constraints on hybridisation agents. Currently PNA probes and structured targets such as Hairpin and Quadruplex are under quantitative analysis for kinetics and thermodynamics of hybridisation.

4.2.3 PNA Complex Stability

Duplexes between PNA and DNA or RNA are in general thermally more stable than the corresponding DNA-DNA or DNA-RNA duplex[38,39]. The sequence dependence of the stability is, however , more complex than that found for DNA-DNA complexes because of the inherent asymmetry of duplexes. In fact PNA-DNA duplexes show significantly increased stability, when more purines are present in the PNA strand. Thus on the top of dependence on G-C content , the stability of PNA-DNA duplexes also depends on the purine fraction of the PNA strand as expressed in the thermal stability of PNA-DNA duplex[40].

$Tm_{pred} = 20.79 + 0.83x\ Tm_{nn\ DNA} - 26.13 \times f_{pyr} + 0.44 \times length$

Tm_{pred}= Tm predicted, Tm_{nn}, f_{pyr}= pyriminine content.

(where Tm DNA is the Tm predicted for analogous DNA-DNA duplex according to SantaLucia et al. [41](not including end effects) and f_{pyr} is the pyrimidine content (fraction) of the PNA strand)).

In general it is also observed that that the thermal stability of PNA-DNA duplex exceed that of PNA-RNA which again are more stable than PNA-DNA duplexes.

Very importantly but not surprisingly the stability of PNA-DNA duplex are almost unaffected by the ionic strength of the medium (actually the stability decreases slightly with increasing Na+ concentration due to counterion release upon duplex formation). This is in sharp contrast, of course, to the behavior of the PNA-DNA (or RNA) duplexes, the stability of which decreases dramatically at low ionic strength because of the requirement of counterion shielding of the phosphate backbone[42]. PNA hybridisation kinetics have been studied and the result indicates – that although PNA's can't be considered conclusive no major difference in PNA and DNA hybridisation duplex formation (on rate)[43].

Most importantly PNA DNA-PNA triplex exhibits extraordinary high stability (the thermal stability, for a ten mer is typically up to 70 %). However, the rate of formation of such ternary triplexes is slow giving rise to significant hysteresis of the thermal transition.

4.3. Melting Temperature (*Tm*-Value):

The physiochemical properties are one of the major determinants of the efficiency of the antisense oligonucleotides, the affinity of binding between the oligonucleotides and its target sequence is characterised by melting temperature (*Tm*) of the double stranded nucleic acids. *Tm* is the temperature at which 50% of the double helix is dissociated into single strand. *Tm* depends on the concentration of oligonucleotides and on the properties of the solvent or solvent systems. In case of unmodified oligonucleotides estimation of *Tm* value can be done by Wallace rule[6,7] which holds good for double stranded DNA with perfect base pairing at high salt concentration.

$$Tm = n\ (2^{\circ}C) + m\ (4^{\circ}C)$$

Where n = number of dA.T base pairs and m = number of dG.C base pairs.

In case of dG.C base pairs stability increases due to the formation of three hydrogen bonds between the base pairs, thus under physiological conditions (37°C, low salt concentration) at least twelve base pairs are required to achieve reasonably stable hybridisation of the oligonucleotides with the target sequence. In practice, a 20-mer oligonucleotide with average base pairing may be assumed to have a *T*m of 54 °C in 0.1M NaCl solution[8]. The thermodynamic properties like δH and δS for the binding of the oligonucleotides with polynucleotides can be can be calculated by Damle formulation[9].

$$1 / Tm = \delta S / \delta H + 2.3\,R / \delta H \log C_M$$

C_M = concentration of free oligonucleotides at T = *T*m

Watson-Crick base pairing permits the formation of double strand between DNA·DNA; DNA·RNA and RNA·RNA with the stability of the double strand decreasing in the order RNA/RNA >RNA/DNA >DNA·DNA[10]. The extent of double strand formation can be checked by gel migration analysis[11] because the mobility of double stranded molecule moves different from that of the corresponding single strand on PAGE.

Modification of oligonucleotides may result in an increase/decrease in *T*m. For eg. if there is alteration in internucleotide bonds by substitution into the phosphate center, the factors that determine *T*m are; the electronic nature (charge), the steric requirement and the absolute stereochemical arrangement of the substituents.

*T*m depends on the ionic buffer used[11] however, this dependence is only with phosphodiester compounds and not with phosphotriester analoges[12]. Second, the stoichiometry of the hybridisation is often different from 1:1 so that the hybrid molecules undergoing dissociation are not always equivalent. Sarin *et al.*[13] have reported that impure methylphosphonate oligonucleotides show a broad *T*m-curve. Tidd *et al.*[14] found a 1:1 complex on sequence specific hybridisation of a 9-mer methylphosphonate with a 20-mer oligonucleotides. It has been reported[13] that unmodified 20-mer methyphosphonate oligonucleotides have *T*m of 55°C which decreases to 51°C when there are four phosphonate residues and changes to 51°C with 18-phosphonate residues. In case of phosphorothioates Tm is considered less for A·T pairs the *T*m considerably less than the G·C pairs[15,16]. The position of phosphorothioate also decreases the *T*m viz. thaiosphorothioates at 5' against the pyrimidine residues lowers *T*m while against purine residues increases the *T*m[16,18].

Thus the binding affinity of an antisense oligonucleotides can be observed from the fall in biological activity as the assay temperature increases. *T*m of large natural DNA molecules ranges from 80-90°C irrespective of particular length, although it depends on G·C content.

Intercalating agents also affect the *T*m. Helene *et al.*[19] reported that acridine residues at the 3' end increase *T*m from 33.5 to 47.3°C. the length of alkyl chain connecting the acridine to the antisense oligonucleotides had a crucial effect on the duplex stability. Introduction of modified bases that

can form more stable Watson-Crick base pairs also affects the *T*m. Diaminopurine (DAPu) forms three hydrogen bonds with thymine, whereas the natural partner adenine is able to form only two hydrogen bonds[20]. The pyridopyrimidine bases published by Inoue *et al.* similarly pair with guanine more strongly than does natural cytosine and thus stabilises the duplex[21]. Furthermore, attachment of relatively large molecules like nucleases to the 3' end of oligonucleotides slightly lowers the *T*m[22].

Alteration in sugar moiety shows considerable effect on *T*m. It has been reported that a 9-mer-2'-deoxyoligonucleotide on hybridisation with a complementary oligoribonucleotide has a *T*m of 41°C. In case of oligoribonucleotides or 2'-O-methyloligoribonucleotide the same sequence shows a *T*m of 50 or 54.3°C respectively[23]. A drastic positive change in *T*m was found by Imbach *et al*[24,25]. for oligonucleotides that have the α-anomeric nucleosides incorporated in place of natural β-isomer as in case of α-d(G2T12G2)·RNA duplex melts at 53°C while the corresponding β-anomeric duplex melts at only 27°C. The stabilisation of the hybridisation appears to be less favorable for purine-rich α-anomeric oligonucleotides than for pyrimidine-rich sequences[26]. Some dephospho modification also lowers *T*m very greatly. It is impossible to determine *T*m for oligonucleotides having diisopropylsiloxane[27] or carbamate[28] bridges. However, Summerton reported[29] a dramatic increase in *T*m when the carbamates are expanded to morpholine ring. Similarly in case of guanylated homooligomers *T*m measurements is no longer possible, since it tends to degrade[30]. Letsinger *et al*[31,32] used cationic oligonucleotides to increase binding affinity for natural anionic polynucleotides.

4.4. Hybridisation Stringency

The successful use of nucleic acids as probes for sequences of interest therefore depends upon certain reaction conditions which are in turn determined by the physical properties (ie. length and sequence) of the probe. This leads to the concept of stringency of hybridisation[1], one increases the stringency by lessening the likelihood of non-homologous annealing. This can be done by simply increasing the temperature of incubation bearing in mind that rate of hybridisation/annealing is maximal at about Tm 25°C, and too high a temperature results in very slow annealing. An acceptable compromise is to anneal at a standard temperature (eg. 65°C), and then wash the annealed and immobilised hybrid molecules to varying degrees of stringency. The extent to which one should wash can be assessed by repeated autoradiography, if the probe is ^{32}P-labelled, or by repeated color assay of replicates in the case of non-radioactively labelled probe. Washing stringency may be increased by varying the ionic strength (from 1.0M NaCl to 0.02M), or varying the temperature (ambient to 65°C). One may also include SDS or other detergent in wash and in hybridisation buffers in order to decrease non-specific attachment of probe to the adsorptive membrane. For this reason a blocking or pre-hybridisation buffer is normally used before and

during the annealing reaction, to block adsorptive sites on the membrane not occupied by target nucleic acid. This normally consists of buffer salts, detergent, protein, inert polymer material, and DNA.

It is possible to include various other constituents in annealing buffers, designed to increase the hybridisation rate, or the stringency, or both. *Formamide* is a helix destabiliser, and enables one to decrease annealing temperature: the presence of formamide decreases the Tm as shown:

TFm = Tm - 0.61(%formamide, w/v)

It is most often used in annealing reactions using RNA as target or probe, and especially with ds RNA hybrids, as these have high Tms, which necessitate elevated reaction temperatures. Standard conditions using formamide would be 42°C with 50% formamide content in the annealing buffer. Formamide also decreases the rate of annealing, so one normally includes substances like dextran sulphate a polyanionic polymer as "molecular exclusion agents" to decrease the volume of solvent available to the probe. Polyethylene glycol is a far cheaper and equally effective substitute for increasing reaction rate. Too high a concentration of DS (Dextran sulphate) or PEG (poly ethylene glycol) raises "background" or non-specific probe attachment to unacceptably high levels. Their effectiveness is also directly proportional to probe length, and they are useless when oligonucleotides of less than 50 nucleotide in length are used as probes.

4.5. Antisense Properties of PNA

The excellent hybridisation properties of PNA and especially the fact that PNA-DNA (or RNA) complexes are stable at low ionic strength at which the DNA (and RNA) secondary structure is resolved, has allowed the development of several techniques for isolation and detection of nucleic acids for analysis in genetic diagnostics[45].

Although this is not the intention to give the elaborate account for the ongoing development in gene therapeutics based on PNA oligomers, it is fair to say that PNA does possess many of the properties desired from an antisense agent. It binds strongly and with excellent specificity to complementary m- RNA. It has very high biological stability, and targeting of specific m-RNA with PNA have been shown to inhibit translation of this m-RNA. This has been demonstrated in several biological systems in vitro, and the introduction of various novel methods for improved delivery of PNA to eukaryotic cells has recently also allowed good efficacy in cell culture ex vivo as well as in few cases in vivo[46-49]. Several reviews have been published in this area[50-52].

4.6. Present work

A survey of literature shows, that PNA-DNA duplex is more stable confirming the greater affinity of the PNA strands towards the DNA strand. This may be attributed to the fact that PNA strand having a neutral backbone is not repulsed by the DNA strand during the hybridisation resulting in comparatively more stable PNA – DNA duplex. Although the sequence taken here in the present

work is having a single PNA monomer (A*, where adenine is the nucleobase), even then it has to be seen that how it is affecting hybridisation and thus the Tm of duplex.

In the present work two strands of the duplex used are

5'-*flu*-AAT GGA T-3' (*flu* = fluorescein, labelled with fluorescein) and

5'-*rh*-A*TC CAT T-3' (*rh* = rhodamine, labelled with rhodamine and modified sequence having PNA monomer A*, adenine is nucleobase here)

Since we had planned to assay the hybridisation using two techniques i.e. Tm and FRET (Fluorescence resonance energy transfer) therefore, labelling of both the strands was done. FRET studies have been described in chapter 5.

The fluorescein unit is attached at the 5'-of the sequence (5'-*flu*-AAT GGA T-3) by using 5-aminopentanol as linker. The fluorescein was first attached with 5-aminopentanol unit and the free hydroxyl terminus was phosphitylated. In the last coupling cycle of DNA sequence amidite was coupled to give 5'-*flu*-AAT GGA T-3' (detail is given in chapter 3).

5'-*rh*-A*TC CAT T-3' is a labelled and modified sequence, the sequence was synthesised using phosphoramidite approach (detail is given in experimental section of chapter 3).

The A* unit represented the PNA monomer having adenine as nucleobase. To this 5'-A*TC CAT T-3' fluorescent group attached is rhodamine. The rhodamine was first attached with a two carbon linker using ethanolamine. The free -OH terminus was further used to generate its phosphoramidite by using pyridinium trifluoroacetate and N,N,N',N'- bis tetraisopropyl phosphoramidite. The usual procedure for preparing amidite building blocks involve phosphitylation of free hydroxy group with 2-cyanoethyl-N, N-diisopropylchlorophosphoramidite in presence of activator 1H tetrazole. Since, 2-cyanoethyl-N, N, N', N'-diisopropylphosphoramidite (bis reagent) is fairly stable and less expensive, therefore, it has been used in the present work.

Since this complementary sequence of 5'-*flu*-AAT GGA T-3 has a modified PNA (Peptide nucleic acid) monomer A*, therefore for monitoring the effect of modification on hybridisation, sequence 5'-*flu*-AAT GGA T-3' was hybridised with its complementary labelled and unmodified sequence 5'-*rh*-ATC CAT T-3'. This unmodified sequence 5'-*rh*-ATC CAT T-3' was synthesised with phosphoramidite approach (synthesis is given in experimental section).

In hybridisation studies mainly phosphate buffers are used at pH 7.0. To maintain stringency condition, sodium chloride was added to the hybridisation buffer. Monovalent cation, Na^+, influences the equilibrium constant and the rate of duplex formation, because they dissipate the negative charge of phosphate. Generally, as the Na^+ concentration increases to 0.2 M, the equilibrium shifts towards duplex formation and the formation rate increases. Furthermore, NaCl affects the interaction between nucleic acid chain and non-ionic aromatic hydrocarbon, such as fluorescein and rhodamine. These effects depend on whether or not these molecule are conjugated

to the chains through covalent linkage. The higher the concentration of Na^+, stronger is the interaction when the chains are single stranded, and the weaker it is when double-stranded. Hybridisation studies were carried out taking at 0.6 O.D scale.

(1 $O.D_{260}$ = 33 μg for single stranded DNA and 50 μg of double stranded DNA, O.D is defined as amount of oligonucleotide when dissolved in a volume of 1.0 mL, results in an absorbance of 1.0 when measured at 260 nm in a 1 cm path length quartz cuvette.).

The melting curves of each hybrid (modified, labelled and unmodified, labelled) were obtained by determining the absorbance at 260 nm (A_{260}) of the solution as a function of temperature. It took 5 min. for the solution to reach temperature equilibrium after each 5^0C incremental increase. The oligonucleotides were taken in 1: 1 ratio in a cuvette of path length 1 cm . The mixtures of oligonucleotide were heated upto 65^0C and then gradually cooled upto 5^0C. In our experiment duplex formation from 0% to 100% occurred at 5 and 65^0C respectively. Absorbance readings were recorded (at 260 nm) and graph was plotted (melting curve 4.7 & 4.8).

4.7 Result and Discussion

In the present course of work, hybridisation of 5'-*flu*-AAT GGA T-3'and its complementary modified sequence 5'-*rh*-A*TC CAT T-3' and unmodified 5'-*rh*-ATC CAT T-3'has been studied. These all sequences were synthesised using phosphoramidite approach.

Step	Reagents and Solvents	Function	Time (min)
1	Anhydrous acetonitrile	Wash	2
2	3% Trichloroacetic acid in dichloromethane	Detritylation	0.5-1.5
3	Anhydrous acetonitrile	Wash	2
4	Nucleotide, ^1H-tetrazole, anhy. Acetonitrile	Coupling Step	15
5	Repeat step 1-4 until the sequence is complete		20
	In the last cycle rhodamine phosphoramidite was coupled		10
6	0.1M iodine/pyridine/water (90/5/5)	Oxidation of Phosphoramidite	

TABLE 4.1 Phosphoramidite synthesis cycle

Since all the sequences were attached with solid support (LCAA- CPG), therefore for cleavage from the solid support sequences were incubated with 30% aq.ammonia at 55^0C for 16 hrs. The free oligonucleotides were obtained after removing the ammonia in vacuuo. For purification of oligo's from impurities such as protecting groups, benzamide, isobutrylamide , failure nucleosides and salts oligo's are precipitated with isopropanol. Subsequently purified by HPLC, using anion exchange column.

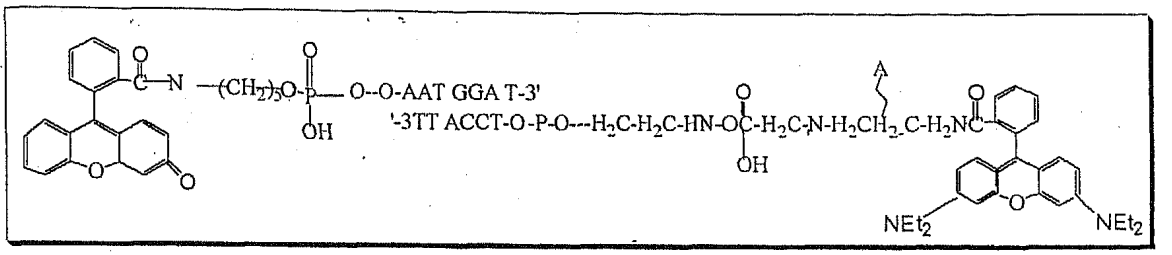

Fig. 4.7. Digramatic presentation of duplex

Sequences were dissolved in a buffer containing 0.1M sodium chloride, 0.01 M potassium dihydrogen phosphate, 0.01 M, sodium hydrogen phosphate with pH adjusted to 7.0. All the three the sequences were kept at 65^{0}C for 2 min. to ensure the complete unwinding of oligo and then it was cooled gradually to 5^{0}C (annealing). The process was repeated twice[53]. The melting curves

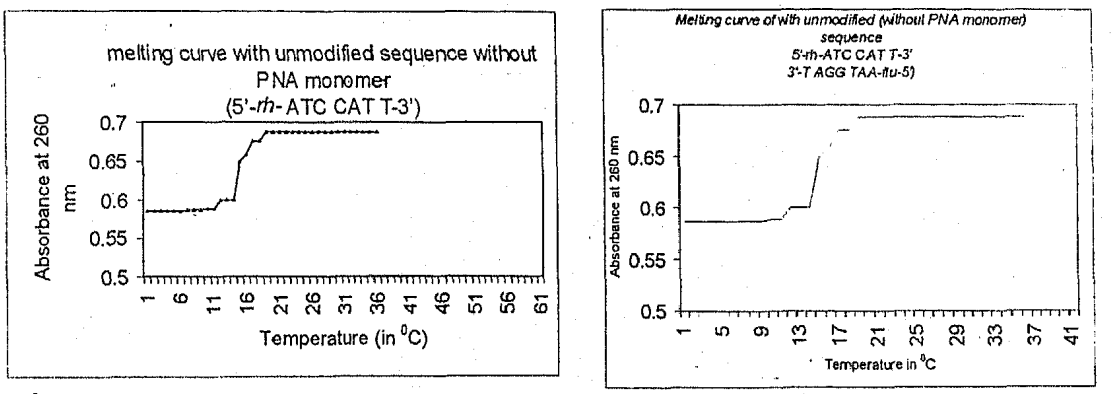

FIG. 4.8. Melting curves with modified, labelled and unmodified, labelled sequences

were studied by recording the change in absorbance at A_{260} with respect to temperature.

Temperature was increased from 5^{0}C to 65^{0}C at a rate of 0.5^{0}C/min. The process was repeated (cooling and heating from 5^{0}C to 65^{0}C) to ensure compatibility in results. Alternatively hybridisation of complementary modified (i.e. with PNA monomer) and unmodified (al natural nucleotides) both sequences were also studied. The respective melting curves for the hybrid sequences are shown in figure 4.8 & 4.9

These hybridised modified, labelled complementary sequences show the melting temperature of 19^{0}C, whereas, the melting temperature of unmodified, labelled complementary sequences was 17.5^{0}C.

As is evident from the results, there is a difference of 1.5^{0}C in the melting temperature of the two sequences, differing only in the presence of the one peptide monomer with adenine as nucleobase. Although the presence of the two dye molecules i.e. rhodamine and fluorescein at the two opposite

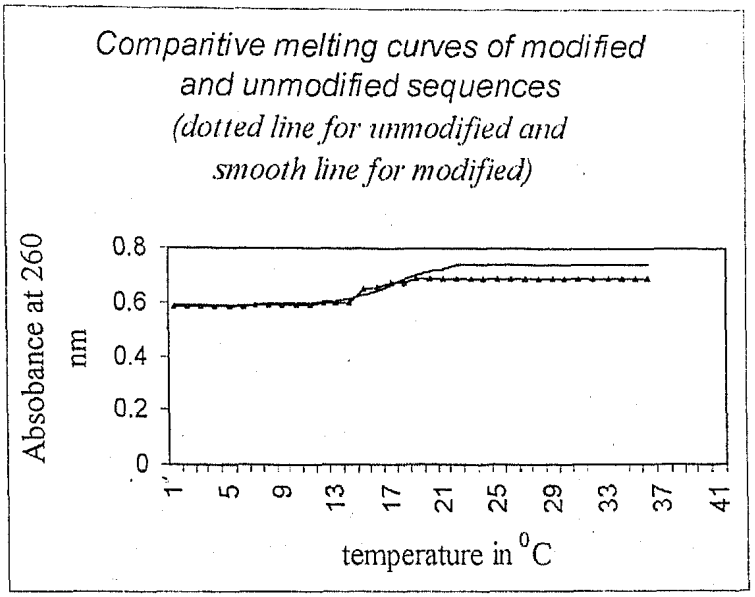

FIG.4.8 Comparative melting curves of modified and unmodified sequences

ends of duplex might contribute significantly to the melting behavior, it will stand neutralised since the standard also carries the same. Therefore, the absolute value of the difference in Tm may not be reliable the fact that a measurable difference of 1.5^{0}C exists is sufficient to infer that even one monomeric unit of PNA is sufficient to give stability to the duplex.

4.8 Materials and Methods

All Solvents used were of Qualigens analytical grade, which were further purified and dried prior to use. 2-Cyanoethyl-N, N, N`, N`-diisopropylphosphoramidite (bis reagent) and pyridinium trifluoroacetate were obtained from ISIS pharmaceuticals. Synthetic oligonucleotide was obtained from Hybridon Inc. USA. 1H-tetrazole and trichloroacetic acid were purchased from Sigma Chemicals company.

The absorption studies were carried out on Hitachi 220S UV-VIS spectrophotometer. The melting profile taken on Hitachi 220S Spectrophotometer attached with HAAKE DC 5 refrigerated circulating water bath with facilities for temperature programming. HPLC was done on LKB-DBF, Pharmacia, and using RPC C_{18} column using 0.01 m ammonium acetate bufer in 80:20 water : methanol gradient. All the glass-wares used were autoclaved. Double distilled and autoclaved water was used for preparation of buffer.

4.9 Experimental

4.9.1 Preparation of Buffers

Phosphate buffer (pH 7.0) : Phosphate buffer was prepared by dissolving NaCl (0.1 M, 580 mg) , K_2HPO_4 (0.01M, 1.136 mg), and $NaHPO_4$ (0.01M, 1.41 mg), in 100 ml of triple distilled autoclaved water.

4.9.2 Synthesis of deoxyoligonucleotide sequence 5'-*rh*-ATC CAT T-3'

I. Synthesis of N-(β-hydroxyethyl)-rhodaminamide:

Rhodamine, (2 mM, 958mg) was dissolved in dioxane (15mL). To its stirred solution p-nitrophenol (2.4mM, 333.9 mg) was added followed by DCC (2 eq.) and DMAP (0.05 eq.). After 2h ethanol amine was added in equimolar ratio with further addition of 2 eq. of DCC. The reaction mixture was stirred for another 5 hr. at r.t. completion of reaction was monitored on tlc with a new spot [Rf.= 0.8, rhodamine,] has Rf of 0.6 in pure DCM). The reaction mixture was evaporated in vaccuo and then loaded on a short silica column (80-120 mesh; 6x2 cm.). The desired product was eluted with 40: 60 hexane: DCM, yield = 60%.

i.a. N-(β-ethyl-O-phosphoramido)-rhodaminamine:

N-(β-hydroxyethyl)-rhodaminamide (1.5mM) was suspended in DCM (15mL). To it 2-Cyanoethyl-N, N, N', N'-diisopropylphosphoramidite (bis reagent) (3mM, 1mL) was added followed by pyridinium trifluoroacetate (3mM, 600mg) at ambient temperature under argon. After 4 h the reaction mixture was checked on tlc. (DCM irrigating solvent). The product spot was found at (Rf= 0.6). The reaction mixture was concentrated in vaccuo and purified on silica by column chromatography.

ii.Covalent attachment of phosphoramidite of Rhodamine to 5'-ATC CAT T-3':

The CPG loaded thymidine was taken in a functionalisation vessel having frit and an uninterrupted argon supply was maintained. The sequence was synthesised with standard phosphoramidite approach[94]. The CPG was washed with dry acetonitrile and the 5`-O-DMTr –group was deprotected by treatment with 3% trichloroacetic acid for 3 min. Stepwise coupling yield was calculated by estimating the trityl color spectrophotometically and was found in the range of 90-92% for all the 6 couplings. After detritylation step column was washed twice with dry acetonitrile. The rhodamine amidite was dissolved in dry acetonitrile and was mixed with equimolar amount of ^{1}H-tetrazole and added to the vessel. A coupling time of 20 min. was allowed, followed by washing with acetonitrile. The sequence was oxidised using I_2 in THF/ Pyridine/ water (90: 5:5) for 15 min. The column was again washed with acetonitrile.

iii. Calculation of loading of nucleosides on LCAA-CPG

Loading of Nucleosides = A498 X Vol. / 76 x 1000 / wt. (μM) of starting resin

Absorbance (495 nm) of trityl solution	=	2.310 OD		
Vol. Of trityl solution	=	5 ml		
Total absorbance	=	2.310 x 5	=	11..550
Therfore, loading of nucleosides	=	11.550/ 76 x 1000/ 4		
	=	11550/ 304		
	=	38.4 μM / g CPG		

Calculation of overall yield (Y_0)

For DNA sequence (5'-ATC CAT T-3')

$$= \frac{\text{Total OD} \times 100}{[(15.4 \times nA) + (8.8 \times nT)] \times \mu M / g \text{ CPG}}$$

$$= \frac{1125.772 \times 100}{[(15.4 \times 2) + (8.8 \times 3)] \times 2}$$

$$= \frac{112577.2}{[(15.4 \times 2) + (8.8 \times 3)] \times 2}$$

$$= \frac{112577.2}{132}$$

$$= 93\%$$

iv. Deprotection of Oligonucleotide from solid support

Oligomer 5'-*rh*ATC CAT T-3' bound to solid support, LCAA-CPG was taken in vials and treated with aq. ammonia (25%) in a Haake K 15 thermostate bath at 55^{0}C for 15 hr. This treatment causes the removal of base protecting groups and subsequent removal of support.

v. Iso-propanol precipitation

After CPG cleavage free oligonucleotide was taken in 1 ml of iso-propanol, and centrifuged at 10, 000 r.p.m. for 1 min. Iso-propanol was decanted without disturbing the DNA pellet. This DNA pellet was further dissolved in 1 ml of hybridisation buffer.

vi. Hybridisation studies.

After cleavage from solid and purification oligos were taken in hybridisation buffer. Sequences were taken in a cuvette of 1 cm path length and their O.D. was fixed at 0.6. Two complementary sequences were further mixed in equal amount at same O.D.

Complementary sequences were kept at 65^{0}C for 30 min. to ensure the complete unwinding of oligo then it was gradually cooled to 5^{0}C. This process was repeated twice. The melting curves were studied by recording the change in absorbance at 260 nm.

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CHAPTER-5

FRET studies of the duplex of two complementary heptamer 5'-fluAAT GGA T-3' & 5'-rhA*TC CAT T-3'

5.1 Fluorescence

Light is composed of photons and each photon is a 'packet' (or quantum) of energy. When a photon strikes a molecule, it imparts, some of its energy to it. This energy is momentary stored in the chemical structure of that molecule. As a result of this absorption of energy, the molecule becomes temporarily unstable i.e it is said to reach an excited state. The wavelength of light that are capable of inducing this excitation in a molecule are called its excitation spectrum.

In order for a molecule to stablise and return to its ground state, it had previously absorbed this energy must somehow be released. Usually absorbed energy is released as heat. Certain molecules (when hit by light of specific wavelength) release very little of the energy imparted to them as heat. Instead, they release energy by emitting photons, that is they emit light. This phenomenon is called fluorescence and the molecule that posses the ability to fluoresce are called fluorophores, which are generally dyes.

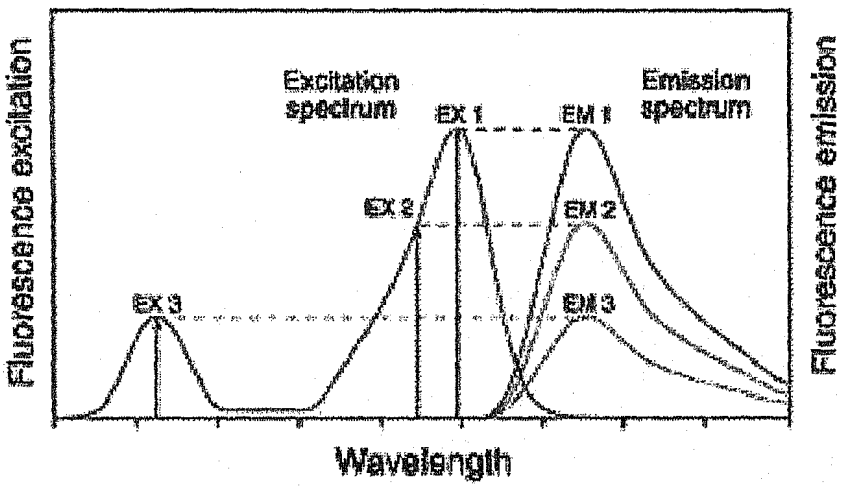


FIG. 5.1 Fluorescence Excitation and Emission Spectrum

Fluorescence is the result of three-stage process:

- **Excitation**

An excitation photon, $h\nu_{EX}$, is supplied by an external source as an incandescent lamp or laser. The energy of this photon is absorbed by the fluorophore, causing it to become excited. It is said to have reached its *electronic singlet state*, s_1, from the *ground state* s_0.

- **Transition in Excited state**

The excited state exists only for a short period, typically 1-10 x 10^{-9} seconds. During this time, the excited molecules undergo conformational changes and they are also subjected to multitude of possible interaction with their molecular environment, resulting in partial energy loss. Heat loss, quenching and fluorescence energy transfer are three example of energy loss. The energy of the excited molecule then drops to *elevated energetic state* s_1.

- **Emission of Photons**

A photon $h\nu_{Em,}$ is emitted, returning the fluorophore to its *ground state, S_0*. Due to energy disssipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, then the excitation photon $h\nu_{Ex}$.

The discrete electronic transition represented by $h\nu_{Ex}$, and $h\nu_{Em}$ above apply to a single molecule. For solution based samples, these transitions vary in their magnitude due to energetic transition such as heat loss. Hence fluorescence of a population molecule is characterised by entire spectra (entire range of wavelength), shown in Fig.5.1.

The intensity of fluorescence emission is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength. While the intensity or amplitude of the fluorescence emission and excitation spetra may change, this is not their overall characteristic but these are physical properties of fluorophore.

5.1.1 Fluorescence intensity assay

The purpose of fluorescence intensity assay is to measure the amount of fluorescence emitted from a sample. Therefore, this emitted light is passed through an excitation filter, which reduces this spectrum to a small wavelength range (the bandpass). This filtered light passes through the sample, thereby exciting it and causing it to fluoresce. The emission spectra is composed of many wavelength of light and this spectrum is a physical characteristic of sample. This emission spectra is reduced to a discrete range of wavelength s by the emission filter. Photons that pass through this

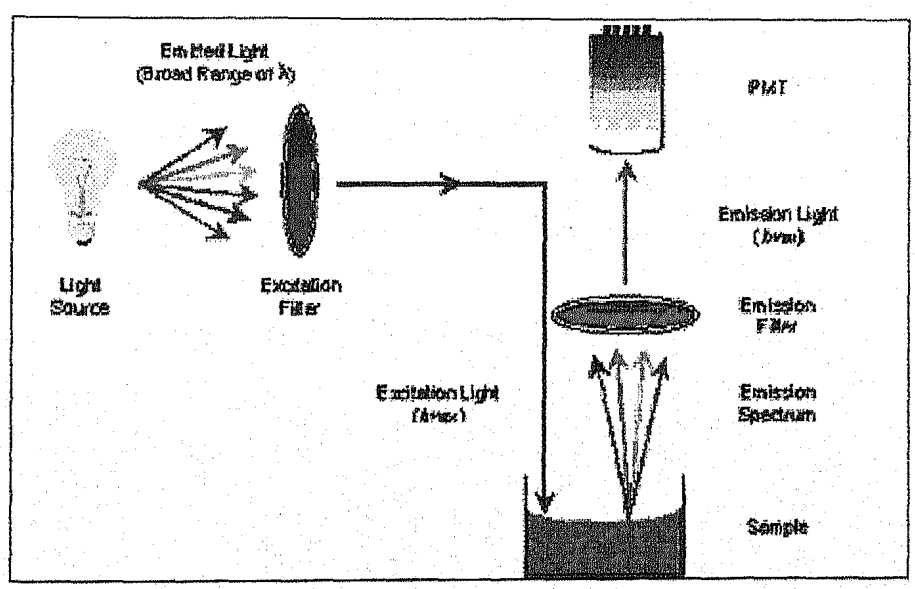


FIG.5.2 Fluorescence Excitation and Emission

filter are then detected by PMT. Output is reported in relative fluorescent units or RFU.

5.1.2 Stoke's Shift

The stoke's effect is the distance between the excitation peak and the emission peak of a fluorophore, expressed in nm. The greater the separation between these two wavelength, the better the results for a fluorescence intensity assay will be. If these two peaks are too close together (less than 35 nm), however, photons from the excitation light will 'bleed' into the range of wavelength measured on the emission side, resulting in optical cross-link. Shown in FIG.5.3, the best result for fluorescence intensity assay are obtained when these two peaks do not overlap.

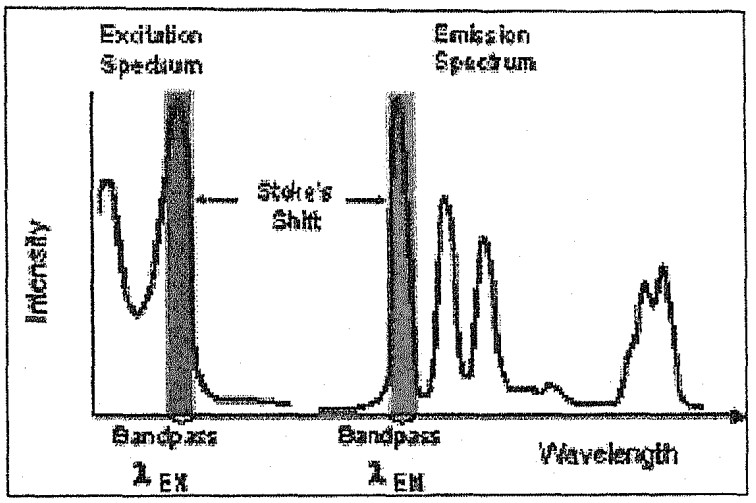


FiG. 5. 3. Stoke's effect

5.2. FRET (Fluorescence Resonance Energy Transfer)

Fluorescence Resonance Energy Transfer (FRET) is a process that shifts energy from an electronically excited molecule (the donor fluorophore) to a neighboring molecule (the acceptor or quencher), returning the donor molecule to its ground state without emission of light (i.e., fluorescence emission). Energy transfer is between different electron states of the donor and acceptor.

Horizontal lines represent discreet electron energy levels for each molecule. Energy levels are labeled as either *singlet states (S)* or *triplet states (T)* with subscripts numbered 0, 1, or 2, representing the ground state, first excited electronic state, or second excited electronic state. Molecules generally reside in its lowest or ground, electronic state, S_0. A molecule may be excited to one of its higher energy levels by any of a number of processes, including light absorption and chemical reaction. Excitation of a molecule is represented in the figure. 5.4 by the arrows pointing upward from the ground state. An excited donor molecule has several routes available to release its captured energy and return to a lower energy state or to the ground state. Released energy can be dissipated to the environment (as light or heat) or transferred directly to another molecule, which

captures that energy and in turn itself moves to a higher energy state. These routes are marked by arrows pointing downward from the excited states; straight lines represent light emissions and

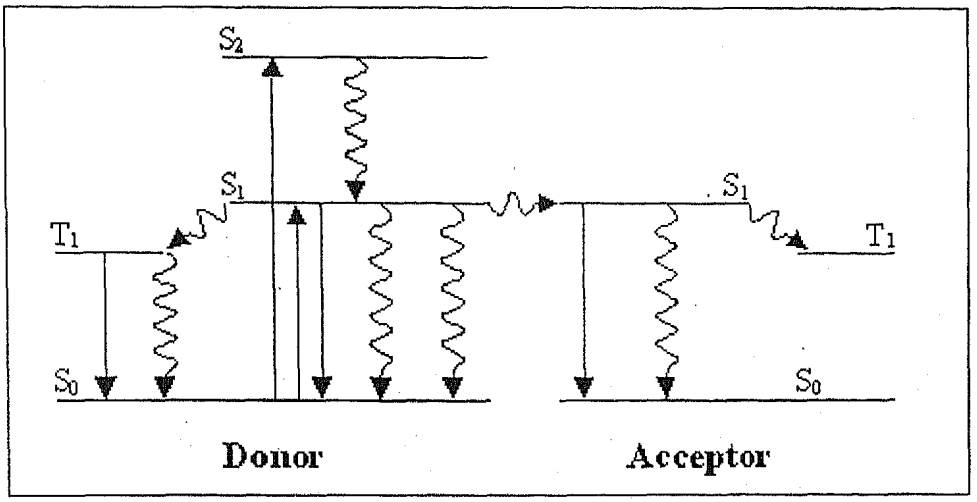


Fig. 5.4. Energy Transfer between Donor and Acceptor

wavy lines represent energy conversion without light emissions.

1. Internal energy conversions. Molecules excited into their second or higher excited states are rapidly de-excited without light to the *first excited state*, S_1, i.e. without interacting with other molecules. Internal conversion from S_1 to S_0 can also be rapid but in a luminescent molecule it is slow enough that is de-excitation by light emission is competitive. Intersystem crossing from S_1 to the triplet state, T_1, also occurs.

2. Light emissions. Light released by the transition from S_1 to S_0 is fluorescence emission. Light released from the *triplet state* T_1, which generates phosphorescence, can also occur but is less common. When a second fluorescent molecule (i.e., a quencher or FRET acceptor) is in physical proximity to an excited fluorophore, new paths for de-excitation become available.

3. Dynamic quenching. This process only occurs when the product of quencher concentration and quenching rate constant is high.

4. Static quenching. This process results from the formation of a non-emissive complex between the donor fluorescent molecule and the quencher. Static quenching reduces the donor fluorescence intensity.

5. FRET. FRET can occur when donor and acceptor molecules are in close proximity but do not require actual physical contact. In the process of FRET, de-excitation of the donor molecule is linked to excitation of the acceptor molecule. In the figure, FRET is represented by the de-excitation pathway leading from the S_1 level of the donor to the S_1 level of the acceptor. Photons of light are not involved. Once excited, the

acceptor can undergo de-excitation by the same emissive and non-emissive processes described for the donor.

5.2.1 Primary conditions for FRET

1. The primary requirement for FRET is that the energy lost by de-excitation of the donor molecule, S_1-S_0, be matched by the energy required for excitation of the acceptor. In other

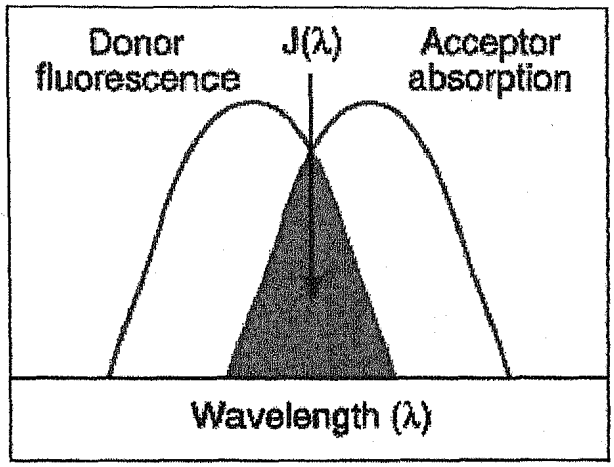


FIG.5. 5. Absorption of acceptor and emission of donor

words, the absorption spectrum of the acceptor molecule must overlap the emission spectrum of the donor molecule, as shown in Fig. 5.5.

2. Donor and acceptor molecules must be in close proximity (typically 10-100 Å). FRET is a distance-dependent energy transfer between the electronic excited states of two dye molecules. The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by FRET) is defined by the Förster radius (Ro).

$$R_0 = [8.8 \times 10^{23} \cdot \kappa^2 \cdot n^{-4} \cdot QY_D \cdot J(\lambda)]^{1/6} \text{ Å}$$

where κ^2 = dipole orientation factor (range 0 to 4; $\kappa^2 = 2/3$ for randomly oriented donors and acceptors)
QY_D = fluorescence quantum yield of the donor in the absence of the acceptor
n = refractive index
$J(\lambda)$ = spectral overlap integral (see figure)
$= \int \epsilon_A(\lambda) \cdot F_D(\lambda) \cdot \lambda^4 d\lambda \text{ cm}^3\text{M}^{-1}$

where ϵ_A = extinction coefficient of acceptor
F_D = fluorescence emission intensity of donor as a fraction of the total integrated intensity

3. Donor and acceptor transition dipole orientations must be approximately parallel.

Donor/Acceptor Pairs: In general, donor and acceptor are different dyes, each having unique spectral properties. Normally, a fluorophore will release light at its characteristic emission wavelength following excitation. When two suitable fluorophores are in proximity within the

distance defined by the Förster radius. FRET will prevent fluorescent emission from the higher energy group. Instead, energy is transferred to the lower energy group, exciting the acceptor, and leading to fluorescence emission at a lower energy wavelength characteristic for the acceptor. If donor and acceptor are the same dye, FRET can still occur and can be detected as fluorescence depolarization. Non-fluorescent acceptors exist which will accept energy from a donor without any resulting fluorescence emission. These acceptors as a group are known as "dark quenchers", and include Dabcyl, QSY, and BlackHole dyes.

FRET is highly efficient within the Förster radius of the donor/acceptor pair (which is often in the 50 - 60 Å range), making it useful over distances encountered within many biological macromolecules. Further, since FRET is dependent on the inverse sixth power of the intermolecular separation, efficiency dramatically falls as donor/acceptor distance exceeds the Förster radius, making it an extremely sensitive indicator of intermolecular distance. Thus, FRET is an important technique for investigating a variety of biological phenomena where markers that track physical proximity are necessary or useful.

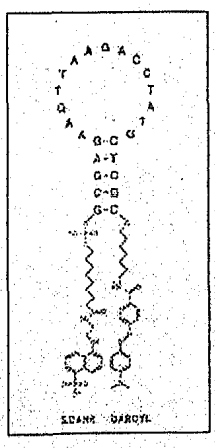
5.2.2 Applications of FRET

- **TaqMan 5'-nuclease assay**

In TaqMan 5'-nuclease assay two fluorescent dyes (such as Fam and Tamra) are covalently linked by DNA residues in an oligonucleotide probe. When the higher-energy fluorophore (Fam) is excited at 488 nm, instead of the expected fluorescence emission at 520 nm, the captured energy is transferred to the lower energy fluorophore (Tamra) and is emitted at 580 nm (FRET has occurred); the Fam signal is quenched. Using the fluorescein/rhodamine reporter/quencher combination, FRET can occur even when the groups are separated by 25-30 bases of DNA. During the course of a TaqMan assay, the two fluorophores are physically separated from each other by the 5'-exonuclease action of Taq DNA polymerase - after which 488 nm stimulation results in visible Fam emission at 520 nm (quenching is released).

- **Molecular Beacons**

Molecular Beacons are another variation on the FRET-based nucleic acid probe paradigm. In this



case, a dark quencher (most commonly Dabcyl) is placed at one end of the DNA probe and a reporter dye is placed at the opposite end. The probe is designed such that a target-specific hybridization domain is positioned centrally between short sequences (unrelated to the target) that lead to hairpin formation. In the native state, the Molecular Beacon forms a hairpin structure with the reporter and quencher groups directly adjacent. When hybridized to the complementary target sequence, the hairpin structure unfolds and the reporter and quencher separate. Dabcyl has a relatively short Förster radius and will not quench the reporter dye in the open configuration.

- **RNase Alert**

A new method to detect RNase activity was recently developed by RNase Alert employs a fluorescence-quenching oligonucleotide probe to detect the presence of RNase activity. A fluorescein reporter group is connected to a fluorescence-quencher by several RNA residues. The precise sequence has been optimized for maximum sensitivity in detecting a wide variety of RNase activities. Upon cleavage of the RNA, the reporter and quencher separate and a fluorescent signal is revealed.

- Substrates for enzyme kinetic studies[1]
- Structure and conformation of proteins[2]
- Spatial distribution and assembly of protein complexes[3]
- Receptor/ligand interactions[4]
- Immunoassays[5]
- Probing interactions of single molecules[6]
- Structure and conformation of nucleic acids[7]
- Detection of nucleic acid hybridization[8]
- Primer-extension assays for detecting mutations[9]
- Automated DNA sequencing[10]
- Distribution and transport of lipids[11]
- Membrane-fusion assays[12]
- Membrane-potential sensing[13]
- Fluorogenic protease substrates[14]
- dicators for cyclic AMP[15]

5.3. Dye labelled oligonucleotides

Fluorescent dyes can be attached to oligonucleotides on the 3'-end, 5'end or even to internal residues. Multiple additions of the same or different fluorophore can be placed on a single oligo. A variety of dyes can be conjugated to oligos directly during oligo synthesis using dye-phosphoramidites or dye-CPG derivatives.

oligonucleotides post synthesis (for example, attaching a dye-NHS-ester to an amino modified oligo).

Dual-labeled probes usually have a 5'-reporter dye, such as Fam, Tet, or Hex and a 3'-quencher group. such as Tamra or Dabcyl. The 3'-group can be attached directly at the time of synthesis using a derivatized CPG (Tamra-CPG or Dabcyl-CPG) or can be attached post synthesis using NHS-ester chemistry (react a 3'-amino modified oligo with Tamra or Dabcyl-NHS-ester). Probes made using dye-CPG need only a single RP-HPLC while probes made using NHS-ester chemistry require two RP-HPLC purifications. The former approach have relatively high yield, but the later approach generates probes that have slightly lower background and greater sensitivity and are recommended for more demanding assays.

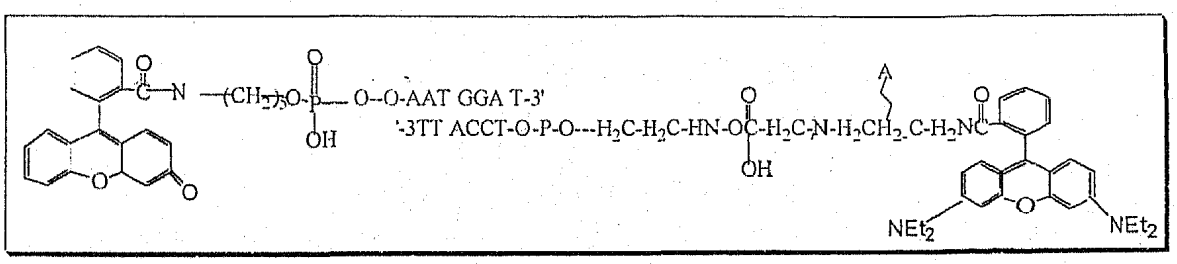
5.4. Present work

As described earlier (in chapter 4) we have characterised and assayed the hybridisation of the heptamer duplexes 5'-*flu*-AAT GGA T-3' and 5'-*rh*-A*TC CAT T-3' with Tm (melting temperature). FRET is another technique for assessment of binding of duplexes. In the present work we have explored this technique (FRET) for assesment of hybridisation.

The two sequences selected for FRET studies are,

5'-*flu*-AAT GGA T-3' (*flu*= fluorescein labelled) and

5'-*rh*-A*TC CAT T-3' (*rh*= rhodamine labelled, A* is modified PNA monomer containing adenine as nucleobase)



Diagrammatic presentation of Hybridisation

Since for FRET studies a distance of 70A^0 is maximum (3.4 A^0 is the distance between two bases of a dinucleotides) therefore, a 7-mer is chosen for the studies (total length 23.8 A^0 calculated).

The fluorescein unit is attached at the 5'-of the complementary sequence by using 5-aminopentanol as linker. The 5-aminopentanol linker was attached at carboxyl moiety of fluoresein and the free hydroxyl end of the linker was used for phosphorylation (to synthesise amidite), which will further couple to give 5'-*flu*-AAT GGA T-3'. Fluorescence studies of this sequence was done in phosphate buffer (Fig.5.7B).

5'-*rh*-A*TC CAT T-3' is a modified sequence where the 5'-TC CAT T-3' was synthesised using phosphoramidite approach (detail is given in chapter 4). The A* unit represented the PNA monomer having adenine as nucleobase. To this 5'-A*TC CAT T-3' fluorescent group attached is rhodamine. The rhodamine was first attached with a two carbon linker using ethanolamine. The

free OH- end was further changed to its amidite by using pyridinium trifluoroacetate and N,N,N',N'- bis tetraisopropyl phosphoramidite.

After the synthesis of the sequence 5`-A*TC CAT T-3` this rhodamine amidite was coupled using phosphoramidite chemistry. Sequences were deprotected from CPG using 30% aq. Ammonia. The sequence was further purified as described in chapter 4. Since we wish to FRET studies therefore, we have chosen rhodamine as the fluorescent molecule which exhibits the property of a acceptor if it comes in close proximity of fluorescein, which acts as donor.

Since we are characterising hybridisation of duplex with FRET and out of these two sequence one contains a modified and labelled sequence 5`-*rh*-A*TC CAT T-3`, we have also carried out a blank experiment with 5`-*rh*-ATC CAT T-3' which is labelled but unmodified (A used here is deoxy nucleoside). This sequence was also synthesised with phosphoramidite chemistry and further amidite of rhodamine was coupled with 5`- (synthesis is described in chapter 4).

We have characterised FRET with rhodamine and fluorescein for nucleic acid assay under homogenous condition. We are using fluorescein- rhodamine because, fluorescein-rhodamine pairs has a large Forster radius (-55^{0}A) (See table. 5.1).

Donor	Acceptor	Forster Radius R_0 (A^0)
Fluorescein	Rhodamine	55
IAEDANS	Fluorescein	46
EDANS	DABSYL	33
Fluorescein	Fluorescein	44
BODIPY-Fl	BODIPY-Fl	57

Table.5.1. Typical values of Forster radius

In general FRET based experiments can determine distance that are within ± 50% of the forster radius i.e. 25^{0}A-75^{0}A in case of fluorescein rhodamine. These primer have a maximum absorbance at 490 nm and 542 nm respectively. Different values of excitation and emission of fluophores, their amidites and labelled oligo's are given below. (Table.5.2)

Dye, primer	**Excitation**	**Emission**
Fluorescein	496 nm	525 nm
Fluorescein amidite	492 nm	520 nm
5`-*flu*-AAT GGA T-3'	490 nm	516 nm
Rhodamine	543 nm	580 nm
Rhodamine amidite	540 nm	565 nm
5`-*rh*-A*TC CAT T-3'	542 nm	565 nm

Table.2. Different values of Excitation and Emission

Fluorescence studies have been carried out in buffer of pH 7.0. The buffer used have 0.1M sodium chloride, 0.01 M potassium dihydrogen phosphate and 0.01 M sodium hydrogen phosphate (pH 7.0). Fluorescence studies were carried out on 0.6 O.D scale.

5.5 Result and Discussion

Synthesis of defined sequences of nucleic acids and the attachment of variety of fluorescence labels at selected positions of the oligonucleotides (both at internal nucleotide sequence position and on the strand end) has become convenient and straightforward. This spectacular advancement has opened up many possibilities for making up detailed physical measurement on nucleic acid structures. The results of fluorescence studies on well defined oligonucleotide has furnished valuable information about the structure of even complicated nucleic acid complexes. Because fluorescence can be observed conveniently under a variety of different experimental circumstances, these results on well-defined models system can be applied advantageously to aid

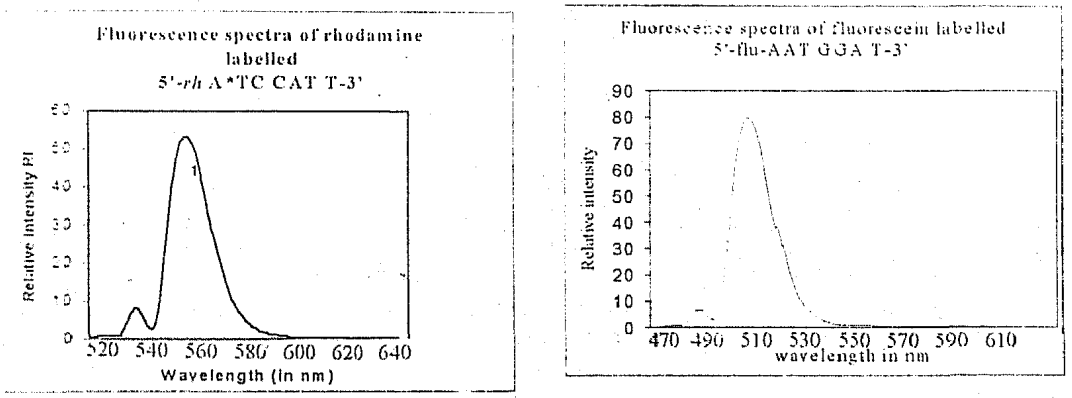


FIG.5.7 Fluorescence spectra of sequences 5'-*flu*-AAT GGA T-3' and 5'-*rh*-A*TC CAT T-3'

in the interpretation of subsequent fluorescence measurements made on more complex molecular structure and biological systems involving nucleic acid.

FRET measurement on duplex with seven base pairs has been done. First of all the absorbance of fluorescence emission spectra of 5'-*flu*-AAT GGA T-3' and 5'-*rh*-A*TC CAT T-3' were taken separately. 5'-*flu*-AAT GGA T-3' and 5'-*rh*-A*TC CAT T-3' shows maximum absorbance (λ_{max}) at 490 nm and 542 respectively. These sequences were excited at 490 nm and 542 nm to determine the emission wavelength. The emission wavelengths of the 5'-*flu*-AAT GGA T-3' and 5'-*rh*-A*TC CAT T-3' were 516 nm and 565 nm respectively. Excitation and emission pattern of both the sequences is shown in FIG.5.7.

Fluorescence spectra were again recorded by exciting the mixture (5'-*flu*-AAT GGA T-3' and 5'-*rh*-A*TC CAT T-3') at the excitation wavelength of fluorescein i.e. 490 nm. The solution having the two sequences were mixed at room temperature i.e. 25^{0}C in equimolar ratio or O.D. The

hybridisation mixture was kept for 30 min. to stablise. The experiment was repeated twice to confirm the result. (FIG.5.8). This peak is evident of the FRET signal (545-560 nm). Calculated value of the emission wavelength of rhodamine is 565 nm. Found value of the emission wavelength of rhodamine is in range of 545-560 nm.

The results of the blank (unmodified sequence 5'-*rh*-ATC CAT T-3') was found to be same, therefore it was inferred that PNA monomer have no significant effect on hybridisation studies indicated by FRET signal.

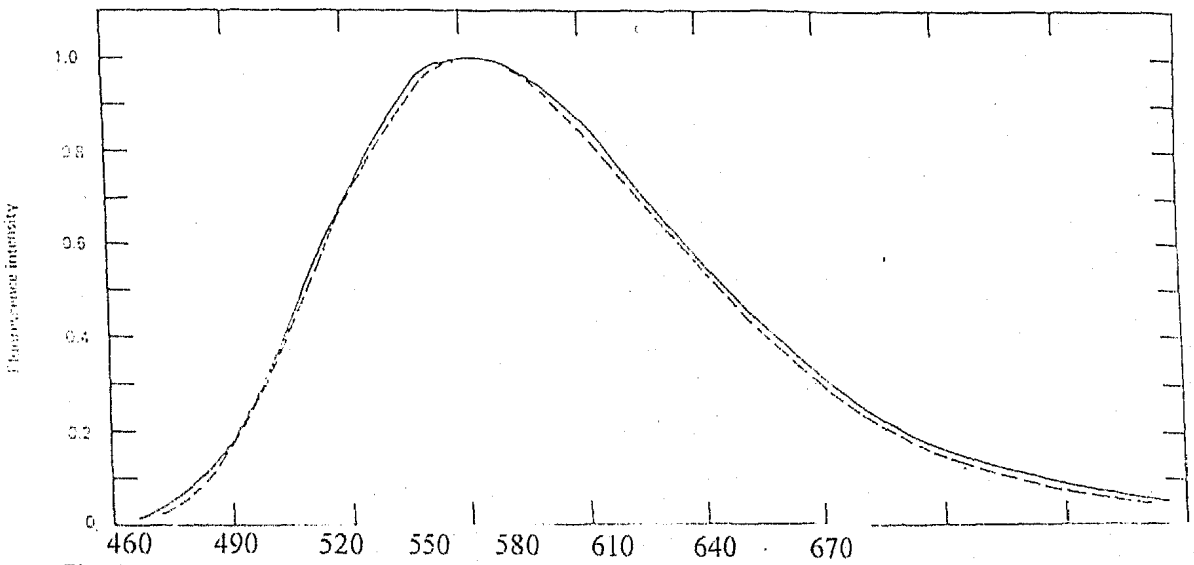


Fig. 5.8: Fluorescence spectra of duplex 5'-*flu*-AAT GGA T-3' and 5'-*rh*-A*TC CAT T-3'.

(broken line represents unmodified sequence 5'-*rh*-ATC CAT T-3' and smooth line, modified sequence 5'-*rh*-A*TC CAT T-3'.

5.6 Experimental

5.6.1 *Preparation of Buffers* .

Phosphate buffer (pH 7.0) : Phosphate buffer was prepared by dissolving NaCl (0.1 M, 580 mg) , K_2HPO_4 (0.01M, 1.136 mg), and $NaHPO_4$ (0.01M, 1.41 mg), in 100 ml of triple distilled autoclaved water.

5.6.2 UV-Absorbance and fluorescence studies:

- *Fluorescein:*

All the absorbance and fluorescence studies of fluorescein were carried out in phosphate buffer at fixed O.D. i. e. 0.6. λ_{max} of fluorescein was at 496 nm. Fluorescence spectra of fluorescein was recorded by exciting the fluorophore at 496 nm. Emission of fluorescein was at 525 nm.

- *Fluorescein amidite:*

λ_{max} of fluorescein amidite was at 492 nm. Solution of amidite in phosphate buffer was taken in a cuvette of I cm path length, and O.D. was fixed at 0.6. Fluorescence spectra of fluorescein amidite was recorded by exciting the fluorophore at 493 nm and emission was found at 520 nm.

- *5'-fluAAT GGA T-3':*

Absorbance of this primer was found at 490 nm and further for the fluorescence studies it was taken in a cuvette of 1cm path length. On exciting the fluorophore at 490 nm its emission was recorded at 516 nm.

- *Rhodamine:*

λ_{max} of rhodamine was 543 nm. For the fluorescence studies solution of rhodamine in phosphate buffer having pH 7.0 was taken in cuvette of 1 cm path length . Emission of rhodamine was recorded at 580 nm.

- *Rhodamine amidite:*

λ_{max} of rhodamine amidite was 540 nm. For fluorescence studies fluorophore was excited at 540 nm and emission was recorded at 565 nm.

- *5'-rhA*TC CAT T-3':*

maximum absorbance of this primer was 542 nm. For emission spectra fluorophore was excited at 542 m and emission was recorded at 565 nm.

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